APPENDIX A

carefully-conducted, large-scale study of the long-term effects of ascorbic acid on blood pressure in patients with hypertension. Such a study should be done before this treatment can be recommended.

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Antenatal corticosteroids: is more better?

Sir—The Jan 22 commentary by G N Smith and colleagues[1] is timely because although the use of antenatal maternal steroids to reduce death and disability in pre-term infants is well established, a culture of blame is developing around obstetricians who fail to prescribe steroids to women at risk in the 10 days or so preceding delivery. These potentially dangerous drugs have been widely used and their use is increasing, possibly with a relative diminution of benefit. The question of adverse effects on development should not be addressed in premature survivors, in whom there are many confounding variables, but in infants born at term after single or multiple doses of steroids have been given. There are many such infants aged up to about 6 years. While awaiting the results of large multicentre studies, which are aiming to answer the question, a quick case-control study may, or may not, provide a rapid resolution of major fears. Our own attempt to set up such a study 2 years ago foundered on poor record linkage between pharmacy and maternity data systems at a time when the latter were being upgraded. Other units with better linkage systems might undertake such a trial.

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1 Smith GN, Kingdom JC, Penning DM, Mathews SG. Antenatal steroids: is more better? *Lancet* 2000; 355: 251–52.

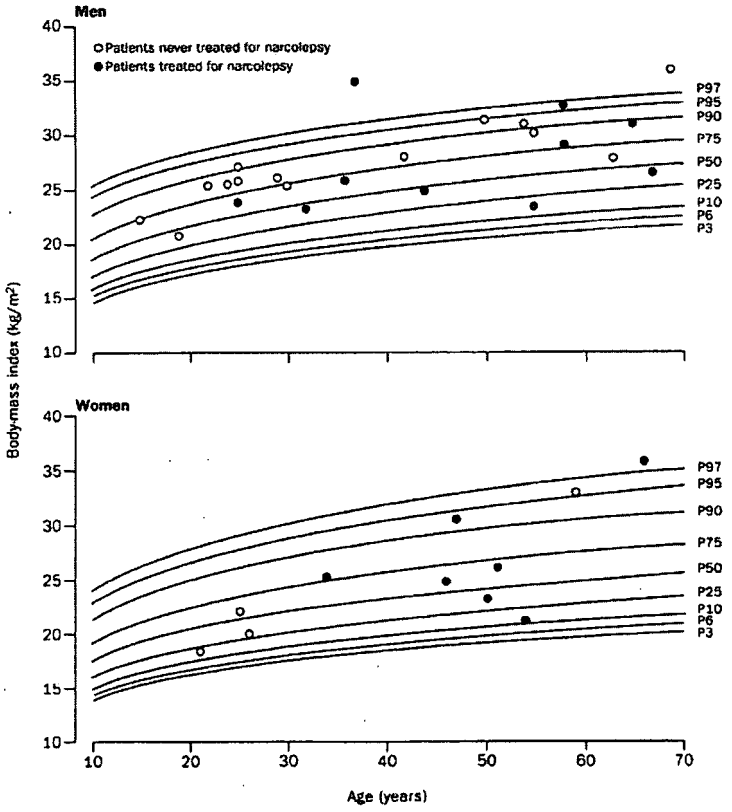
Increased body-mass index in patients with narcolepsy

Sir—Seiji Nishino and colleagues (Jan 1, p 39)[1] showed that patients with narcolepsy have reduced concentrations of hypocretin (orexin) in their cerebrospinal fluid. Because hypocretins stimulate food intake in animal models, Siegel hypothesised[2] that patients with narcolepsy should be lean, rather than obese. However, in an earlier report Honda and colleagues[3] suggest that in patients with narcolepsy the frequency of obesity and of non-insulin dependent diabetes is increased compared with a non-healthy control group of psychiatric patients.

To gather more reliable information, we retrospectively identified all patients of 14–70 years who had narcolepsy, and who were treated in the Max-Planck-Institute of Psychiatry, Munich, Germany, between 1988 and 1999 (n=45). We selected all patients with: excessive daytime sleepiness; cataplectic attacks; multiple sleep-onset REM (rapid eye movement) periods (SOREMs); and who were HLA-DR2 positive. In these 35 patients (24 men, 11 women) we recorded the body-mass index (BMI). At the time of height and weight measurements 18 patients (mean age 36·3 [SD 17·3] years) had never been treated pharmacologically for the disease. 17 patients (mean age 48·5 [12·6] years) had previously received tricyclic antidepressants, psychostimulants, or combinations of these drugs before.

The BMIs were plotted into BMI-percentiles representative of the German male and female population, respectively (figure).[4] The distribution of the BMIs differed significantly from the rectangular distribution on the 0–100 interval (Kolmogorov-Smirnov test). This was apparent both in male (mean BMI percentile 75·6 [23·0]) and female (61·2 [32·5]) patients. The mean BMI-percentile of those patients who had never been treated for narcolepsy (74·8 [25·0]) was in the same range.

These results suggest that patients with narcolepsy have a higher BMI



Distribution of BMI-values of men (upper panel) and women (lower panel) with narcolepsy in comparison with population-based percentiles

than population controls. This could result from altered eating behaviour or energy homoeostasis. Because no obvious differences between medicated and drug-naïve patients were apparent, we suspect that the higher BMI is linked to the pathophysiology of the disease. This association could arise from a direct pathogenic link that involves hypocretins. Alternatively, it may be a consequence of disease-related behaviour—eg, reduced locomotor activity, increased amounts of sleep, or other aspects of behaviour resulting in a lower energy expenditure. Because it remains unclear whether and how an increased BMI in narcolepsy is associated with decreased hypocretin concentrations,[1] BMI and hypocretin concentrations should be studied in parallel in patients with narcolepsy. Moreover, the association between BMI and hypocretin concentrations should also be explored in patients with other disorders of excessive sleepiness as well as in healthy and obese controls.

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STD research and policy formulation

Sir—We have some concerns regarding the article on management of reproductive-tract infections in Bangladesh, by Sarah Hawkes and colleagues (Nov 20, p 1776).[1] The investigators worked with highly selected women served by an intensive family-planning programme. The women were also married and regular attendants of maternal and child health/family-planning clinics. Further, participants appeared to be older, of greater parity, and more likely to be users of contraceptives compared with those that declined involvement. These concerns can be confirmed by reanalysis of the original data available from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB). These results, available to the investigators, should have been presented in the paper submitted to *The Lancet*. In addition, ICDDRB reported that the risk assessment component—without which syndromic management can not be fully evaluated—was not explored as recommended. In a previous study,[2] Wasserheit and colleagues articulately described why extrapolation of conclusions associated with reproductive health behaviour and services in this population subgroup was not possible. Extrapolation of such data from this study to Bangladesh and the rest of Asia is poor science and a grave mistake.

Indicators	Matlab (ICDDRB intervention area)	Bangladesh
Contraceptive prevalence rate (%)	70	49
Crude death rate (per 1000)	6·6	8·0
Infant mortality rate (per 1000 livebirths)	50	67
Child death rate (1–4 years)	4·5	11·0
Life expectancy at birth	67·7	59·8

Comparison of health indicators between Matlab (ICDDRB intervention area) and Bangladesh[3]

ICDDRB is a state-of-the-art research institute. Researchers from the institute have been working in the vicinity of Matlab for over 25 years. Because of several interventions in this area, in particular the intensive family planning home delivery service, there is a significant difference between Matlab and other parts of the country.

We have reviewed over 60 reports on diagnosis of sexually-transmitted diseases (STD) and management experience from the non-governmental-organisation and research communities in Bangladesh. The conclusions and policy decisions taken differed substantively[4] from those of Hawkes and colleagues. Nevertheless, persistence in presenting and publishing these interpretations have had a strong influence on donors' willingness to fund STD services. The subject is technical and some donor representatives had difficulties grasping the issues. Extensive time and energy has gone into resolving the confusion created. Scientists, consultants, and representatives of: ICDDRB, the Government of Bangladesh, the Bangladesh Rural Advancement Committee, Bangladesh Women's Health Coalition, WHO, Marie Stopes, the Association for Voluntary Surgical Contraception, the Population Council, USAID, and the World Bank became involved. The study by Hawkes and colleagues was reviewed and rejected by the international and national scientists involved locally. STD management in the 5th Health and Population plan remains based on the syndromic approach

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Authors' reply

Sir—Contrary to the claims of Fawzia Rasheed and Enamul Karim, at no point in our paper did we state that Bangladesh or the rest of Asia have a low prevalence of sexually-transmitted infections (STIs). On the contrary, we clearly state in our conclusion that surveys among groups at higher risk in Bangladesh have shown high levels of STIs, and we recommend appropriate interventions among these groups as a first step in achieving STI control. Throughout the paper we have made it clear that these findings may apply in any situation of low prevalence, no matter where its geographical location.

The decision to site the study in the Matlab Health and Research Centre was taken on the grounds that the stated function of Matlab is to be a "learning place [for] operations research [and] programme implementation".[1] The scientific value of research done at the Matlab centre lies in the ability of researchers to carry out high quality interventions research with a view to assessing efficacy and impact before these interventions are replicated on a wider scale. Whilst the family planning success of Matlab is well recognised,

APPENDIX B

Neurobiology of Disease

Expression of a Poly-Glutamine-Ataxin-3 Transgene in Orexin Neurons Induces Narcolepsy–Cataplexy in the Rat

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The sleep disorder narcolepsy has been linked to loss of hypothalamic neurons producing the orexin (hypocretin) neuropeptides. Here, we report the generation of transgenic rats expressing a human ataxin-3 fragment with an elongated polyglutamyl stretch under control of the human *prepro-orexin* promoter (*orexin/ataxin-3* rats). At 17 weeks of age, the transgenic rats exhibited postnatal loss of orexin-positive neurons in the lateral hypothalamus, and orexin-containing projections were essentially undetectable. The loss of orexin production resulted in the expression of a phenotype with fragmented vigilance states, a decreased latency to rapid eye movement (REM) sleep and increased REM sleep time during the dark active phase. Wakefulness time was also reduced during the dark phase, and this effect was concentrated at the photoperiod boundaries. Direct transitions from wakefulness to REM sleep, a defining characteristic of narcolepsy, occurred frequently. Brief episodes of muscle atonia and postural collapse resembling cataplexy were also noted while rats maintained the electroencephalographic characteristics of wakefulness. These findings indicate that the *orexin/ataxin-3* transgenic rat could provide a useful model of human narcolepsy.

Key words: cataplexy; electroencephalography; electromyography; sleep; REM; lateral hypothalamus

Introduction

Narcolepsy is a debilitating disorder, affecting 20–60 per 100,000 adults (Mignot, 1998; Overeem et al., 2001; Beuckmann and Yanagisawa, 2002). A cardinal symptom is excessive daytime sleepiness, manifested particularly as attacks of somnolence at inappropriate times. The latency for rapid eye movement (REM) sleep is also notably reduced, and the presence of pathologically short transitions from wakefulness to REM sleep is a diagnostic criterion for the disorder. Other symptoms of narcolepsy include hypnogogic and hypnopompic hallucinations and cataplexy. Cataplexy is characterized by sudden attacks of muscle weakness, frequently triggered by strong emotions (Bassetti and Aldrich, 1996) with patients remaining conscious during an attack (Scrima, 1981; Billiard, 1985; Gerhardstein et al., 1999; Taheri et al., 2002). The symptoms of narcolepsy have been considered an intrusion of REM sleep-related phenomena into wakefulness, indicating that the disorder may be one of vigilance state boundary control (Saper et al., 2001).

The concurrent discoveries that the autosomal recessive form of canine narcolepsy is caused by a mutation in the orexin receptor-2 gene (Lin et al., 1999; Hungs et al., 2001) and that *orexin*$^{-/-}$ mice exhibit a phenotype similar to human narcolepsy (Chemelli et al., 1999) provided insight into the pathophysiology of narcolepsy. In narcolepsy patients, a dysfunction or loss of orexin-containing neurons in the hypothalamus was subsequently confirmed (Peyron et al., 2000; Thannickal et al., 2000). Orexin-expressing neurons constitute a highly delimited population in the perifornical region of the lateral hypothalamus (LH) (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999; Chou et al., 2001). Two neuropeptides, orexin-A and -B (hypocretin-1 and -2), are derived from the *prepro-orexin* gene and act at two G-protein-coupled receptors, orexin receptor-1 and -2 (de Lecea et al., 1998; Sakurai et al., 1998). In the human, mutations of the *prepro-orexin* or *orexin receptor* genes appear to be extremely rare (Peyron et al., 2000; Gencik et al., 2001). However, undetectable to very low levels of orexin-A neuropeptide in the CSF have been described in most patients with narcolepsy–cataplexy, whereas orexin-A levels of patients presenting with other disorders were comparable with those of healthy controls (Nishino et al., 2000, 2001; Ripley et al., 2001; Mignot et al., 2002). The number of orexin neurons is

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*C.T.B. and C.M.S. contributed equally to this work.

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therefore likely to be diminished in narcoleptic patients (Peyron et al., 2000; Thannickal et al., 2000), although the cause for this apparent neuronal degeneration remains undetermined (Lin et al., 2001; Taheri et al., 2002).

In an attempt to mimic the loss of orexin neurons, we recently created transgenic mice in which orexin neurons expressed a cytotoxic gene product (a truncated form of human ataxin-3) under control of the human *prepro-orexin* promoter (Hara et al., 2001). This resulted in degeneration of orexin-containing neurons and a narcoleptic phenotype. We have now expressed the *orexin/ataxin-3* transgene in the rat, a species more widely used for physiological and pharmacological studies, and we describe here the initial histological and phenotypic characterization of the transgene expression in this species.

Materials and Methods

Generation of transgenic rat lines. The *orexin/ataxin-3* transgene expresses an N-terminally truncated human ataxin-3 protein containing a Q_{77} polyglutamine stretch under control of the human *prepro-orexin* promoter (Hara et al., 2001). The C terminus of the transgene contains a Myc oncogene epitope for histological examination. The transgene was injected into pronuclei of fertilized Wistar rat eggs to generate founder animals, which were bred to produce ten *orexin/ataxin-3* transgenic lines. Two lines were chosen for additional evaluation. Although one line is described here in detail, both lines displayed an essentially comparable phenotype. Lines were maintained in the hemizygous state for these studies. The transgene was propagated in a Mendelian manner, indicating no embryonic lethality. Throughout the study, animals were housed under a constant 12 hr light/dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center and were strictly in accordance with National Institutes of Health guidelines.

Genotyping. PCR was performed on genomic DNA from tail biopsies. Primers used were 5′-GCA GCG GCC ATT CCT TGG-3′ and 5′-CAG CGT AAT CTG GAA CAT CGT ATG GG-3′ against the *orexin/ataxin-3* transgene and 5′-GCA CCG AAG ATA CCA TCT CTC CGG ATT GC-3′ and 5′-GAC TCT GGA TCC GCC CCG GGG CGC TAA AGC-3′ against the endogenous rat *prepro-orexin* gene as an internal control.

Immunohistochemistry. All histological examinations were performed on male hemizygous transgenic rats (N3 back-cross generations into Sprague Dawley) and their male wild-type littermates. Male rats (wild-type and hemizygous transgenic littermates 4, 7, 10, 13, and 17 weeks of age) were deeply anesthetized with chloral hydrate (3.5 gm/kg, i.p.) and transcardially perfused with 150 ml of ice-cold PBS (11 U/ml heparin) and 150 ml of 4% (w/v) paraformaldehyde in PBS, pH 7.4. Brains were rapidly dissected and postfixed for 12 hr in 4% (w/v) paraformaldehyde in PBS, pH 7.4, at 4°C before being cryoprotected by equilibrating them in 30% sucrose in PBS. After cyroprotection, samples were frozen and sectioned (30 μm) with an SM2000R sliding microtome (Leica, Nussloch, Germany). Sections were then stored at 4°C in 0.004% sodium azide in PBS before histochemical staining.

For orexin/Myc double-immunohistochemistry, free-floating sections were rinsed with PBS three times for 5 min, followed by 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidases. Sections were then rinsed again with PBS three times for 5 min. Nonspecific binding sites were blocked with 3% (v/v) normal horse serum (Vector Laboratories, Burlingame, CA) with 0.25% Triton X-100 in PBS for 2 hr at room temperature. After blocking, sections were incubated with Myc monoclonal antibody (Ab) (9E10; 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) against the transgene product for 12 hr at 4°C. As a secondary Ab, biotinylated horse anti-mouse Ab (Vector Laboratories) at a 1:600 dilution in 0.25% Triton X-100/PBS was applied for 2 hr. Subsequently, sections were incubated with horseradish peroxidase streptavidin at a 1:500 dilution in 0.5% Triton X-100/PBS for 1 hr, followed by application of $NiSO_4$-enhanced DAB solution (60 μl of 10% $NiSO_4$ per 2 ml of DAB; Dako, High Wycombe, UK) for 7–10 min at room temperature to create a dark brown-to-black precipitation. Orexin immunohistochemistry (light brown DAB staining without nickel enhancement) was performed as described previously (Chemelli et al., 1999) after Myc staining was completed. Sections were then mounted onto coated slides, dehydrated through an ascending ethanol/xylene series, and embedded using Permount (Fisher Scientific, Houston, TX).

Vigilance state determination. For behavioral evaluation, the control group consisted of male wild-type littermates of transgenic rats (N3 back-cross generations into Sprague Dawley) as well as of age- and weight-matched nonrelated male wild-type Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). At 14 weeks of age, rats (8 hemizygous *orexin/ataxin-3* transgenic rats, 20 wild-type controls) were anesthetized (40 mg/kg ketamine, 4 mg/kg xylazine, 1 mg/kg acepromazine, i.p.) and surgically implanted with recording electrodes under sterile conditions. Miniaturized electrodes were affixed to the skull using a glass ionomer dental cement (Ketac Cem; ESPE, Norristown, PA), such that the electrodes just penetrated the skull and touched the dura. EEG signals were recorded unilaterally from fronto-occipital electrode pairs, positioned 1.5 mm rostral and 1.45 mm lateral from bregma, and 3.1 mm caudal and 1.45 mm lateral from bregma. EMG signals were concurrently recorded from two flexible wires, insulated except at the tips, and implanted bilaterally by blunt dissection into the nuchal musculature. The rats were tethered to a counterbalanced arm (Instech Laboratories, Plymouth Meeting, PA) that allowed virtually unrestricted mobility of the animals and exerted minimal weight. All rats recovered from surgery and habituated to the recording conditions for 3 weeks before recording commenced.

EEG–EMG signals were recorded under controlled conditions (12 hr light/dark cycle; 24 ± 1°C) for three consecutive days as described previously (Chemelli et al., 1999). Signals were digitized at 250 Hz, recorded to hard disk, and subsequently archived to optical media for off-line analysis. Concurrently with EEG–EMG recording, the behavior of the rats was videotaped for the first 4 hr of each dark phase using an infrared light source. For vigilance state analysis, EEG–EMG data were classified visually into 20 sec epochs by two independent observers blinded as to genotype. Standard criteria for rodent vigilance state measurements were used (Radulovacki et al., 1984), and results were summarized in terms of non-REM (NREM) sleep, REM sleep, and wakefulness. Epochs of wakefulness were not discriminated for active and quiet wakefulness. Summary vigilance state data for each rat were averaged over 3 d, before the results were grouped according to genotype, and analyzed by alternate Welch t test. The criterion for rejection of the null hypothesis was $p < 0.05$.

The following vigilance state parameters for the 12 hr dark and light periods were derived from the summary data for each rat before averaging by genotype. Total times spent in wakefulness, NREM, and REM sleep were derived by summing the total number of 20 sec epochs in each state. The number of episodes of wakefulness, NREM, and REM sleep was counts of the number of episodes of that state. Because we adopted a 20 sec epoch duration for vigilance state analysis, the minimum duration for an episode to be included in this analysis was 10 sec. Mean episode durations for wakefulness, NREM, and REM sleep were determined by dividing the total time spent in each state by the number of episodes of that state. Mean inter-REM interval was derived by determining the interval from the start of one REM sleep episode to the start of the next REM sleep episode and calculating the average of these intervals. A minimum of 10 sec of REM sleep was required before an episode was counted.

Mean REM sleep latency was determined by averaging the time elapsed from the beginning of a continuous NREM sleep episode to the beginning of the subsequent REM sleep episode. This calculation was also based on the 20 sec episode duration. Thus, if >10 sec of wakefulness occurred during an NREM sleep episode, the calculation was restarted and the interval was derived from the beginning of the NREM sleep episode that commenced after the intrusion of wakefulness. REM sleep latency as calculated here is thus sensitive to vigilance state fragmentation, because a brief arousal that lasted >10 sec was sufficient to reset the

calculation. Similarly, >10 sec of NREM sleep before the REM sleep episode was required for the latency to be included in the average. Thus, the minimum latency in this calculation was 20 sec, and episodes of direct transition to REM sleep were not counted.

Episodes of abnormal transition from wakefulness to REM sleep are considered a defining characteristic of narcolepsy. For this initial study in the rat, we defined an abnormal REM sleep transition as an REM sleep episode that followed a minimum of 60 sec of wakefulness with <20 sec of intervening NREM sleep. The former criterion ensured that brief arousals just before an REM sleep episode were not counted as abnormal transitions. The total count of such events, defined as the number of episodes of abnormal transition to REM sleep, was determined.

The EEG frequency distribution was analyzed by power spectral analysis [i.e., fast Fourier transform (FFT)] from 1 to 32 Hz using SleepSign 2.0 (Kissei Comtech, Tokyo, Japan). For derivation of power spectra for each vigilance state, FFT data for 150–200 representative artifact-free 4 sec epochs per rat (50 epochs for cataplexy per rat) from the 72 hr continuous EEG recordings were averaged for the transgenic rats and their wild-type littermates. Data were then normalized to a spectral density function by dividing each bin by the total average power of all epochs for that rat over the recording period to allow comparison between individual animals. Statistical analysis was by Student's t test, and the null hypothesis was rejected at $p < 0.05$.

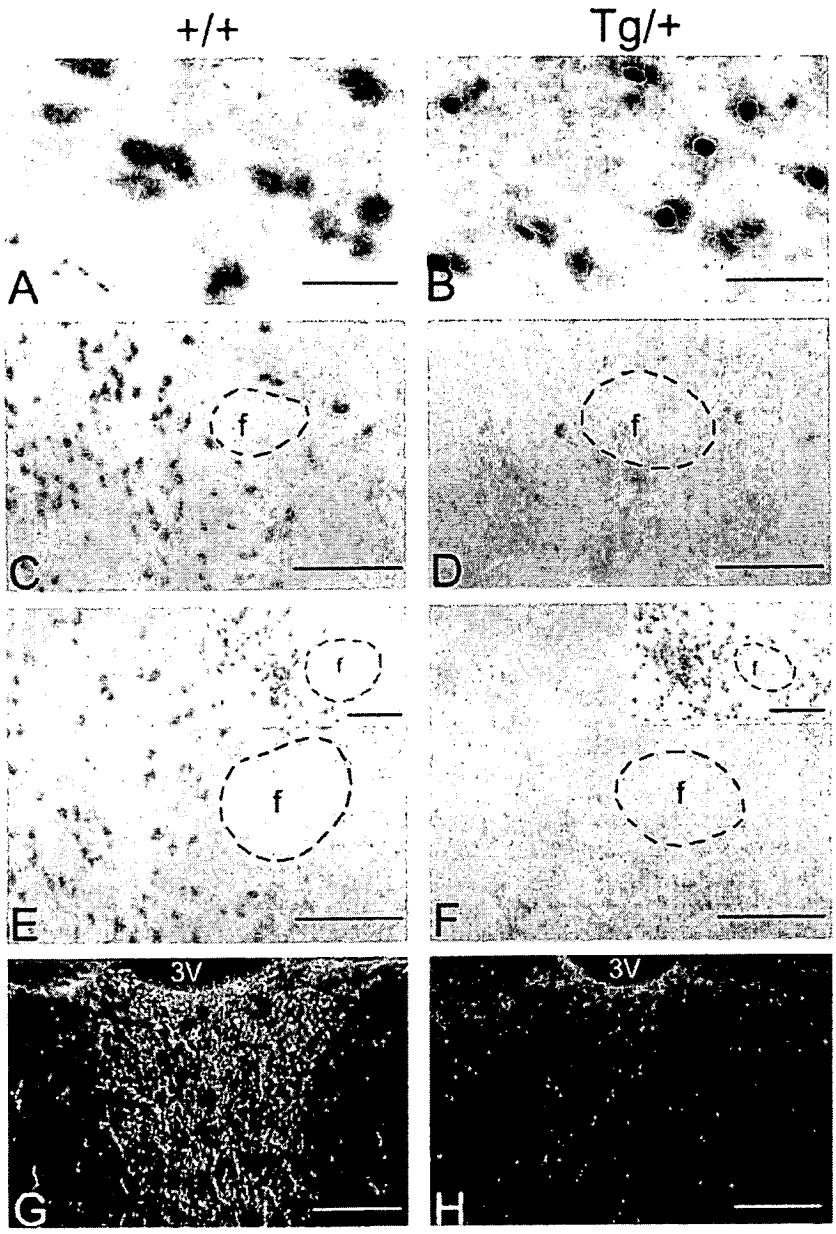

Figure 1. *A–H*, Colocalization of orexin neuropeptide and the *orexin/ataxin-3* transgene product (*A*, *B*) and disappearance of orexin-positive neurons (*C–F*) and projections (*G*,*H*) in *orexin/ataxin-3* hemizygous transgenic rats. The LH region of wild-type rats (*A*) and *orexin/ataxin-3* hemizygous transgenic littermates (*B*) was stained using antibodies against orexin-A (brown) and against the Myc tag of the transgene (black). All transgenic animals showed nuclear staining of the transgene product only in orexin neurons. Wild-type animals showed a normal distribution of orexin neurons in the LH at 4 weeks (*C*) and 17 weeks (*E*) of age. In contrast, *orexin/ataxin-3* transgenic littermates showed a notable reduction of orexin-immunoreactivity by 4 weeks of age (*D*) and a virtually complete loss of immunoreactivity at 17 weeks of age (*F*). No difference in MCH neuronal population in the perifornical area could be found at 17 weeks between wild-type rats (*E*, inset) and their *orexin/ataxin-3* hemizygous transgenic littermates (*F*, inset). Dense orexin-containing projections in the thalamic paraventricular nucleus of wild-type animals (*G*) were undetectable in transgenic animals at 17 weeks of age (*H*). f, Fornix; 3V, third ventricle. Scale bars: *A*, *B*, 40 μm; *C–H*, insets, 200 μm. *A–F*, Bright-field microscopy; *G*, *H*, dark-field microscopy.

Results

Generation of *orexin/ataxin-3* transgenic rats

Expression of the *orexin/ataxin-3* transgene was examined by immunohistochemical staining against the Myc tag at 4 weeks of age. We observed ectopic expression of the transgene outside the LH in 4 of 10 lines; we discarded these lines. In the line described here, the transgene product was colocalized exclusively in orexin-expressing neurons of the LH as verified by double immunostaining against Myc and orexin-A (Fig. 1*A*,*B*). In this line, ectopic expression outside the LH was absent throughout the brain (data not shown). By 4 weeks of age, the number of orexin-expressing cells was notably diminished in the perifornical region of the LH compared with wild-type littermate controls (Fig. 1*C*,*D*). The number of orexin-positive cells continued to decline at 7, 10, and 13 weeks of age (data not shown). At 17 weeks, the age of vigilance state recording, essentially no orexin-positive neurons could be found in the LH (Fig. 1*E*,*F*). However, at this age, immunostaining against Myc revealed picnotic remnants of cell nuclei that were positive for the *orexin/ataxin-3* transgene product in the LH, despite the complete lack of orexin-like immunoreactivity (data not shown).

Orexin-containing neuronal projections were also examined. The thalamic paraventricular nucleus is an area that, *inter alia*, receives a dense orexinergic innervation (Fig. 1*G*). In transgenic rats, no orexin-positive projections could be found at 17 weeks of age in this thalamic nucleus (Fig. 1*H*). Other brain regions, including the tuberomam-

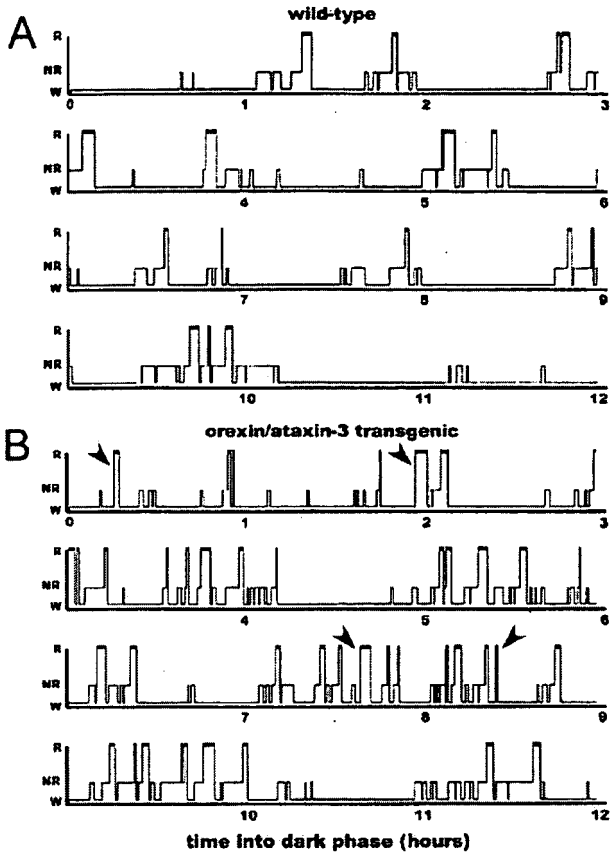

Figure 2. *A*, *B*, Representative dark-phase hypnograms of a wild-type rat (*A*) and an *orexin/ataxin-3* hemizygous transgenic littermate (*B*). The hypnogram of the transgenic animal shows more rapid cycling between vigilance states. It also exhibits direct transitions from wakefulness to REM sleep (indicated by arrowheads). W, Wakefulness; NR, NREM sleep; R, REM sleep.

millary nucleus, central gray, perifornical nucleus, arcuate nucleus, laterodorsal and pedunculopontine tegmental nuclei, locus coeruleus, and raphe nuclei, were also examined and similarly found to be lacking orexin projections.

Neurons expressing melanin-concentrating hormone (MCH) are intermingled with orexin neurons in the LH but form a distinct neuronal population (Elias et al., 1998). No difference in MCH expression was observed between *orexin/ataxin-3* transgenic animals (Fig. 1*F*, inset) and their wild-type littermates at 17 weeks of age (Fig. 1*E*, inset). Thus, the disappearance of orexin expression in the LH was not the result of a generalized lesion or widespread cell degeneration in that area. In summary, the immunohistochemical evaluation of *orexin/ataxin-3* hemizygous transgenic rats indicated a selective disappearance of orexin-containing neurons in the LH, combined with a concomitant loss of orexinergic projections throughout the brain.

Vigilance state characteristics of *orexin/ataxin-3* rats

REM sleep dysregulation

A hallmark characteristic of narcolepsy is dysregulation of REM sleep, and *orexin/ataxin-3* transgenic rats showed marked deviations from the wild-type phenotype in this sleep stage. A representative hypnogram during the dark, active phase in wild-type rats is displayed in Figure 2*A*, and the corresponding hypnogram for a transgenic rat is displayed in Figure 2*B*. Bouts of REM sleep in the wild-type rats were always preceded by an NREM sleep episode that usually lasted for several minutes: the mean REM sleep latency was longer during the dark phase in these rats (2.9 ± 0.2 min vs 3.8 ± 0.2 min for transgenic and wild-type rats, respectively; $p = 0.002$) (Table 1). In contrast, episodes of abnormal transition to REM sleep could be observed in all *orexin/ataxin-3* transgenic rats, primarily during the dark phase, whereas this was found only once in one wild-type control rat during a total of ~1500 hr of recording time. *Orexin/ataxin-3* transgenic rats had 3.8 ± 0.8 episodes showing abnormal transition to REM sleep per 12 hr dark phase and 0.3 ± 0.1 of these episodes per 12 hr light phase. Episodes of abnormal transition were therefore less frequent than normal REM sleep events (42.9 ± 2.8 and 27.9 ± 2.6 REM sleep events during dark and light phase, respectively) (Table 1). The mean duration of these episodes of REM sleep after an abnormal transition was 119 ± 11 and 68 ± 15 sec for the dark and light phases, respectively. Therefore, these durations were comparable with normal REM sleep episode durations (90.5 ± 2.2 and 67.1 ± 4.8 sec during the dark and light phase, respectively) (cf. Table 1).

Figure 3 shows representative traces of EEG–EMG recording from an *orexin/ataxin-3* transgenic rat. Normal transitions from wakefulness to NREM sleep (Fig. 3*A*) were indistinguishable from those in wild-type rats. However, as noted above, abnormal transitions to REM sleep (in this example, a direct transition without intervening NREM sleep) could be observed frequently in transgenic animals (Fig. 3*B*). Power spectral analysis of the EEG did not reveal any differences in the frequency spectra of the vigilance states between *orexin/ataxin-3* transgenic and wild-type rats (Fig. 4*A*, *B*). Furthermore, in *orexin/ataxin-3* rats, the average power spectrum of REM sleep episodes after an abnormal transition was indistinguishable from the corresponding spectrum for those REM sleep episodes that were preceded by NREM sleep (Fig. 4*C*). Thus, an REM sleep episode after an abnormal transition was essentially identical to a normal REM sleep episode with respect to both the EEG frequency distribution and episode duration.

The time spent in REM sleep during the dark phase in *orexin/ataxin-3* transgenic animals was approximately twice that recorded in the wild-type controls (64.2 ± 3.0 vs 32.9 ± 2.6 min for transgenic and wild-type rats, respectively; $p < 0.0001$) (Table 1). This resulted from the increase in the number of REM sleep episodes (42.9 ± 2.8 vs 23.4 ± 1.9 for transgenic and wild-type rats, respectively; $p < 0.0001$). In the light phase, however, overall REM sleep time was significantly less in the transgenic rats than in the wild-type controls (30.6 ± 2.6 vs 50.4 ± 2.6 min for transgenic and wild-type rats, respectively; $p < 0.0001$), resulting from a decrease in the number of REM sleep episodes (27.9 ± 2.6 vs 41.8 ± 2.4 for transgenic and wild-type rats, respectively; $p = 0.001$). In contrast to these differences in REM sleep episode number, the mean duration of REM sleep episodes was not significantly different between the transgenic and wild-type rats in either the dark or light phases (Table 1). The hourly distribution of REM sleep time across the 24 hr also was strikingly different between the genotypes (Fig. 5*A*). In fact, during only 6 hr, primarily clustered around the photoperiod boundaries were the hourly REM sleep times not significantly different between the wild-type and transgenic rats ($p > 0.05$). Therefore, the genotypes not only demonstrated the opposite circadian pattern in the incidence of REM sleep, as also evidenced by the total time spent in this stage during the light and dark 12 hr periods (Table 1), but the transgenic rats also did not show the gradual increase in REM

Table 1. Vigilance state parameters recorded from *orexin/ataxin-3* hemizygous (Tg/+) and wild-type control (+/+) rats

	REM sleep		NREM sleep		Awake	
	+/+	Tg/+	+/+	Tg/+	+/+	Tg/+
24 hr						
Total time[a] (min)	83.3 ± 2.9	94.8 ± 3.6*[b]	663 ± 13	691 ± 15	691 ± 14	651 ± 18
Episode duration (sec)	78.2 ± 2.6	80.9 ± 2.7	231 ± 5	223 ± 9	291 ± 18	238 ± 24
Number of episodes	65.5 ± 3.4	71.0 ± 4.0	174 ± 5	189 ± 11	148 ± 5	172 ± 13
REM latency (min)	5.8 ± 0.3	4.5 ± 0.2**				
Inter-REM interval (min)	22.5 ± 1.2	20.0 ± 1.3				
Light phase						
Total time (min)	50.4 ± 2.6	30.6 ± 2.6**	493 ± 6	497 ± 7	175 ± 6	191 ± 9
Episode duration (sec)	73.9 ± 2.8	67.1 ± 4.8	305 ± 8	330 ± 17	135 ± 7	147 ± 13
Number of episodes	41.8 ± 2.4	27.9 ± 2.6**	98.8 ± 2.9	92.2 ± 4.7	78.0 ± 2.4	80.9 ± 5.9
REM latency (min)	7.4 ± 0.3	7.2 ± 0.5				
Inter-REM interval (min)	15.8 ± 0.9	24.5 ± 1.7**				
Dark phase						
Total time (min)	32.9 ± 2.6	64.2 ± 3.0**	170 ± 9	194 ± 17	516 ± 10	460 ± 19*
Episode duration (sec)	86.4 ± 4.8	90.5 ± 2.2	137 ± 5	121 ± 7	490 ± 52	321 ± 37*
Number of episodes	23.4 ± 1.9	42.9 ± 2.8**	75.5 ± 3.9	96.7 ± 6.8*	70.0 ± 3.8	91.5 ± 7.4*
REM latency (min)	3.8 ± 0.2	2.9 ± 0.2**				
Inter-REM interval (min)	42.7 ± 8.5	17.8 ± 1.3**				

[a]Total time spent in each state (minutes), episode duration (seconds), number of episodes, REM latency, and interval between successive REM sleep episodes (in minutes) over 24 hr is itemized separately for the light and dark phases.
[b]Significant differences between (+/+) ($n = 20$) and (Tg/+) ($n = 8$) rats are indicated with two asterisks (**$p \leq 0.01$) or one asterisk (*$0.01 < p < 0.05$). Data are expressed as mean ± SEM.

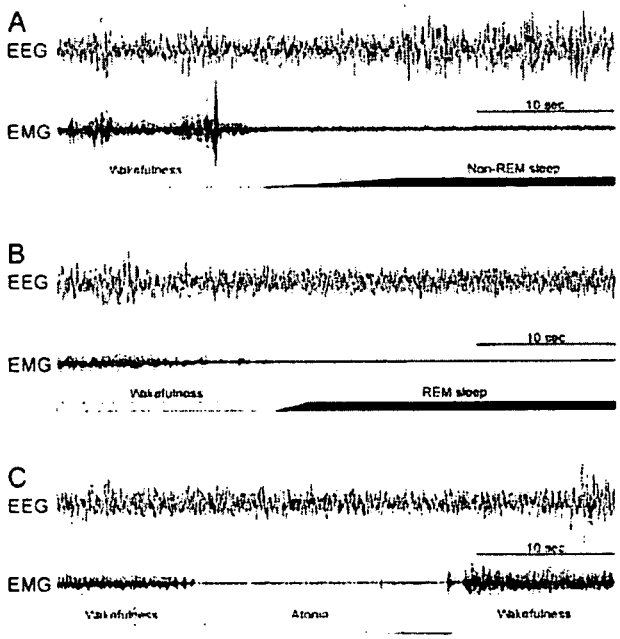

Figure 3. Representative EEG–EMG recordings from an *orexin/ataxin-3* hemizygous transgenic rat. *A*, Normal transition from wakefulness to NREM sleep. The EEG signal increases in amplitude and slows in frequency, whereas neck muscle tone diminishes in the EMG signal. *B*, Abnormal direct transition from wakefulness to REM sleep. The EEG signal starts with the typical mixed-frequency, low-amplitude, wakefulness pattern, which directly gives way to the regular low-amplitude pattern of REM sleep, dominated by θ activity in the 6–10 Hz range, with concomitant complete neck muscle atonia. *C*, Cataplexy-like event. Although the EEG characteristic of wakefulness remains unchanged, a sudden and transient complete neck muscle atonia occurs, after which normal muscle activity suddenly resumes. Note that θ activity in the EEG signal is less than that recorded during REM sleep, and that no visual indications of NREM sleep are apparent during this interval.

sleep time during the 12 hr light period that was a hallmark of REM sleep in the wild-type rats.

Fragmentation of wakefulness and NREM sleep

The hypnogram of a wild-type rat (Fig. 2*A*) showed prolonged periods of wakefulness with occasional sleep episodes. In contrast, *orexin/ataxin-3* transgenic rats showed marked fragmentation of vigilance states during the normally active dark phase, characterized by more rapid cycling between wakefulness and sleep (Fig. 2*B*). This fragmentation during the dark phase is reflected in an increased number of wakefulness episodes (91.5 ± 7.4 vs 70.0 ± 3.8 for transgenic and wild-type rats, respectively; mean ± SEM; $p = 0.03$), NREM sleep episodes (96.7 ± 6.8 vs 75.5 ± 3.9; $p = 0.02$), and REM sleep episodes (42.9 ± 2.8 vs 23.4 ± 1.9; $p < 0.0001$) (Table 1). However, although the transgenic rats had a greater number of wakefulness episodes, the reduced mean episode duration (321 ± 37 vs 490 ± 52 sec for transgenic and wild-type rats, respectively; $p = 0.014$) resulted in less time spent awake (460 ± 19 vs 516 ± 10 min for transgenic and wild-type rats, respectively; $p = 0.03$). We also noted a corresponding tendency toward increased NREM sleep time in the transgenic rats (194 ± 17 vs 170 ± 9 min for transgenic and wild-type rats, respectively; $p = 0.23$) (Table 1). A plot of the hourly distribution of wakefulness time demonstrated that the difference between the genotypes in time spent awake was concentrated primarily at the photoperiod boundaries (Fig. 5*B*). Figure 5*B* also shows that, even in terms of the hourly distribution, wakefulness time during the light period was very similar in both genotypes.

Behavioral analysis

Behavior was monitored for 4 hr by infrared video recording, starting at the onset of the dark phase, while EEG–EMG signals were recorded simultaneously. Subsequent analysis of the video recordings revealed that episodes of abnormal transition to REM sleep occasionally occurred during motivated behavior, such as ambulation or drinking (cf. supplemental video 1, available at www.jneurosci.org). Each abnormal transition to REM sleep was associated with a sudden loss of muscle tone and concomitant loss of posture. Rocking movements along the body axis, as described in *orexin*$^{-/-}$ mice (Chemelli et al., 1999) and in saporin-lesioned rats (Gerashchenko et al., 2001), were not observed. Behavioral arrests associated with these abnormal REM sleep episodes in the transgenic rats were always abruptly terminated with the resumption of full mobility and purposeful behavior. As noted above, the EEG power spectrum for these episodes of REM

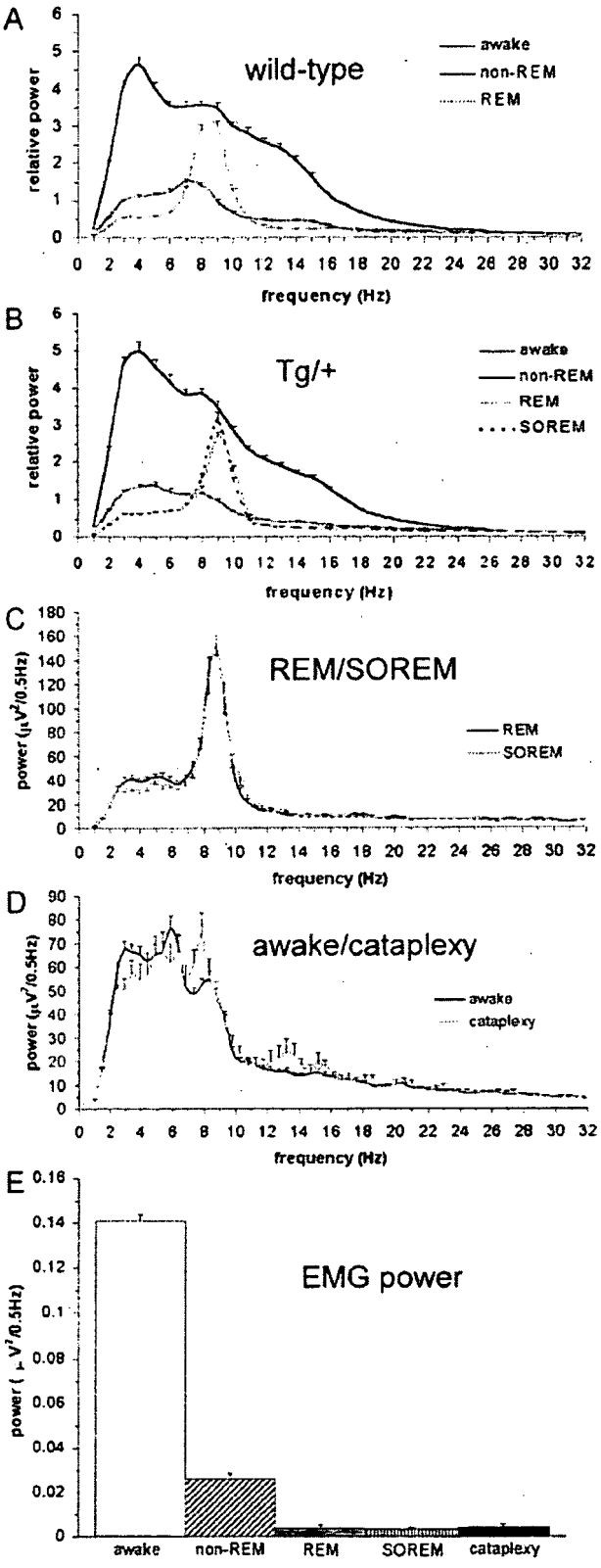

Figure 4. Power spectra of EEG–EMG recordings from wild-type rats and their *orexin/ataxin-3* hemizygous transgenic littermates. *A*, Representative EEG power spectra of wakefulness, NREM sleep, and REM sleep in a wild-type rat. *B*, Representative EEG power spectra of an *orexin/ataxin-3* transgenic littermate. SOREM (i.e., sleep-onset REM) episodes designate those REM sleep episodes that follow an abnormal transition from wakefulness. Note that the mean EEG frequency distribution of SOREM episodes is essentially identical to that recorded during normally occurring REM sleep in the same animal. The signal amplitudes in *A* and *B* have been normalized in each animal to allow comparison across animals. *C*, *D*, Representative absolute EEG power spectra of REM sleep and SOREM episodes (*C*) and of wakefulness and cataplexy-like events in an *orexin/ataxin-3* hemizygous transgenic rat (*D*). *E*, Average EMG signal power recorded during the designated vigilance states from a representative *orexin/ataxin-3* hemizygous transgenic rat. Data are expressed as mean ± SEM.

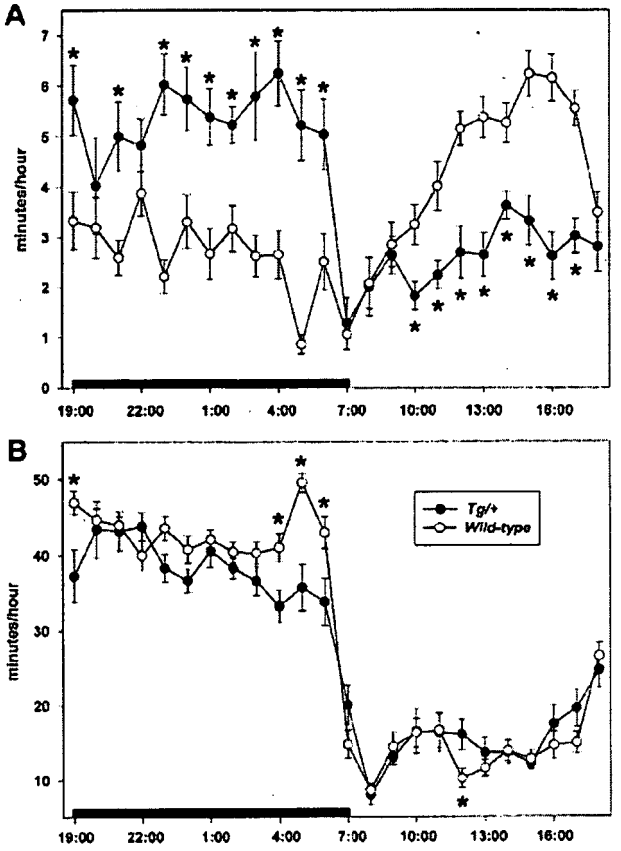

Figure 5. *A*, *B*, Time spent each hour (in minutes; mean ± SEM) in REM sleep (*A*) and wakefulness (*B*) for wild-type rats and their *orexin/ataxin-3* hemizygous transgenic littermates. Significant differences between the genotypes (*t* test; $p < 0.05$) are marked by asterisks. The dark period is denoted by the horizontal bar.

sleep after an abnormal transition was not different from the spectrum recorded during normally occurring REM sleep (Fig. 4*C*).

In addition to typical behavioral arrests associated with abnormal transitions to REM sleep, another type of postural collapse was observed (cf. supplemental video 2, available at www.jneurosci.org). These latter episodes could not be behaviorally differentiated from an episode of abnormal transition by video photography alone. As in abnormal transitions to REM sleep, the rat showed a sudden and complete loss of muscle tone, and purposeful behavior ceased (Fig. 3*C*). However, power spectral analysis of the corresponding EEG and EMG signals revealed that *orexin/ataxin-3* transgenic rats had this loss of muscle tone at a time when the EEG maintained a pattern very similar to that recorded during normal wakefulness (Fig. 4*D*). These arrests occurred both during the dark (4.7 times per 12 hr) and light (2.8

times per 12 hr) phases and were generally short in duration (dark phase, 12 ± 1 sec; light phase, 20 ± 4 sec). As displayed in Figure 4*E*, muscle tone, when compared with either normal wakefulness or NREM sleep, was markedly reduced and became essentially absent during REM sleep, REM sleep after an abnormal transition, and these episodes of behavioral collapse with wakefulness-like EEG. In contrast to human and canine cataplectic events, however, which are often triggered by emotional stimuli (Aldrich, 1992; Riehl et al., 1998), we could not reliably elicit behavioral arrests in the *orexin/ataxin-3* transgenic rats using external emotive stimuli.

Discussion

Although canine and murine models of narcolepsy have been established previously (Mitler et al., 1976; Chemelli et al., 1999, 2000; Hara et al., 2001; Kisanuki et al., 2000, 2001; Willie et al., 2003), human narcolepsy is likely to result from a gradual degradation of orexin neurons and consequent loss of orexin expression. Here, we adapted to the rat the technique for producing *orexin/ataxin-3* transgenic mice, in which orexin neurons were genetically ablated by selective expression of a cytotoxic poly-Q-ataxin-3 protein (Hara et al., 2001). An alternative approach was used by Gerashchenko et al. (2001), who infused orexin-B conjugated to the ribosome-inhibiting cytotoxic protein saporin into the LH of wild-type rats. Orexin-expressing neurons presumably possess orexin autoreceptors, and the infusion of saporin thus resulted in a loss of these neurons and a narcoleptic-like phenotype. However, other LH cell populations that express orexin receptors, including MCH and adenosine-deaminase-containing neurons, were also lesioned by this technique (Gerashchenko et al., 2001). Moreover, a recent electrophysiological study showed that orexin activates orexin neurons via glutamatergic interneurons (Li et al., 2002). Because orexin B-saporin might also ablate these interneurons, this approach cannot be considered a precise model of human narcolepsy, in which orexin neurons appear to be affected in a highly specific way.

The rat model described here thus combines the selective targeting of orexin-expressing neurons and a gradual loss of these cells during ontogenesis, with the advantages of a species that is widely adopted for physiological and pharmacological studies. The most important characteristics of this model are: decreased wakefulness during the normally active dark phase; fragmentation of wakefulness and NREM sleep patterns; shortened REM sleep latency; episodes of abnormal transition to REM sleep; differences in total REM sleep time; and cataplexy-like behavioral arrests, during which the animals show muscle atonia comparable with REM sleep but remain awake as judged by spectral analysis of the EEG. Additionally, vigilance state fragmentation during the dark phase indicates that transgenic rats are unable to maintain prolonged periods of wakefulness. Ongoing activity was more frequently interrupted by bouts of NREM sleep than in the wild-type controls. However, despite these marked differences in sleep during the dark phase, the time spent in wakefulness and NREM sleep over a 24 hr period remained unchanged in the transgenic rat. Overall, these results are directly comparable with vigilance state observations in narcoleptic humans, and we conclude that the phenotype of the *orexin/ataxin-3* transgenic rat closely resembles narcolepsy.

Cataplexy, in particular, is difficult to provoke in a clinical setting in narcoleptic patients, and in most cases, the diagnosis of cataplexy is based on self-evaluation by the patient. The mechanisms of cataplexy have been intensely studied to date in narcoleptic dogs (Riehl et al., 1998; Fujiki et al., 2002). Elicited by emotional stimuli or food presentation, cataplectic attacks in these dogs result in complete muscle atonia, frequently affecting the whole body. Concurrently, the animals are awake and remain aware of their surroundings (Nishino and Mignot, 1997). Analysis of *orexin*$^{-/-}$ mice also demonstrated the existence of episodes of postural collapse, during which the mice remained conscious as judged by behavioral observation (Willie et al., 2003). Detailed characterization of the abnormal state transitions in the *orexin/ataxin-3* transgenic rat are now required, but we noted similarities between these existing descriptions of cataplexy and a particular type of behavioral arrest in these rats.

The striking difference between the wild-type and *orexin/ataxin-3* transgenic rats in the hourly distribution of REM sleep time throughout the 24 hr period contrasts with the similarity of the corresponding wakefulness times, especially during the light phase. The wakefulness deficit in the transgenic rats during the dark, or active, period is essentially concentrated at the photoperiod boundaries and in particular at the end of this period. This time corresponds closely to the timing, in humans, of maximal circadian alertness, which consolidates wakefulness at the end of the active part of the daily cycle when the homeostatic drive for sleepiness is highest (Dijk and Czeisler, 1994). With a polyphasic sleep pattern and no single consolidated wakefulness bout, a similar alertness signal has not been investigated in the rat. However, our data in the wild-type animals demonstrate, just before the beginning of the sleep period, a significant increase in wakefulness, which is absent after the loss of orexin in the transgenic rats. Importantly, the circadian variation in the orexin signal in rat brain peaks at the same time as this increase in wakefulness, late in the active portion of the daily cycle (Taheri et al., 2000; Fujiki et al., 2001; Yoshida et al., 2001). Our data thus support the proposal that orexin contributes to the daily variation in wakefulness at the end of the active period (Mignot, 2001). Previous corroboration for this hypothesis came from Dantz et al. (1994), who used a forced desynchrony protocol to show that narcoleptic patients have a deficit in circadian wakefulness, even though their circadian pacemaker per se and homeostatic sleep drive are normal. Also, a recent study in the squirrel monkey has demonstrated that the maximal orexin signal in this species corresponds to the timing of maximum circadian alertness at the end of the active period (Zeitzer et al., 2003).

Comparison of the hourly distribution of REM sleep time between the genotypes indicates an apparently continuous effect of the loss of orexin on the expression of REM sleep. Thus, the absence of orexin in the transgenic rats results in increased REM sleep throughout the normally active phase and, consequentially, a reduced homeostatic drive for REM sleep during the light phase. In the wild-type rat, the homeostatic drive for REM sleep during the normal sleep phase is expressed as a gradual increase in the hourly times spent in REM sleep. Significantly, this distribution of REM sleep in the wild-type rats is inversely correlated with the diurnal variation of the orexin signal, which remains high throughout the dark period and reaches a minimum toward the end of the light phase in this species (Taheri et al., 2000; Fujiki et al., 2001; Yoshida et al., 2001). Together, these data suggest that the release of orexin has an inhibitory influence on the appearance of REM sleep.

Importantly, the difference in REM sleep time between the genotypes was attributable to a change in the number of episodes of REM sleep, whereas the REM sleep bout length remained unchanged. This indicates that orexin normally inhibits the onset of an episode of REM sleep but, once initiated, it does not affect the characteristics of that episode. Kiyashchenko et al. (2002) re-

ported that orexin release is highest during active wakefulness and REM sleep. This result left open the possibility that orexin also might be involved in the generation of the REM sleep state. The current data now preclude this possibility and suggest that the link to orexin is through ongoing motor patterns, whether they are expressed as in active wakefulness or inhibited as in REM sleep. We therefore speculate that an important function of orexin is to inhibit the appearance of REM sleep and particularly when the brain state, including cortical activation, is most similar during active wakefulness. For this reason, when orexin is absent, the REM sleep switch appears biased (Saper et al., 2001; Sutcliffe and de Lecea, 2002), and direct transitions from wakefulness to REM sleep can occur.

In summary, our data show that the presence of orexin impacts vigilance state control in two ways. First, it may act as a circadian arousal signal to enhance alertness at the end of the normal wake period when the homeostatic drive for sleepiness is reaching its maximum. Second, it appears to inhibit the onset of REM sleep episodes. Orexin is excitatory to the brainstem monoaminergic cells, which are quiescent during REM sleep (i.e., the REM-off cells) (Hagan et al., 1999; Horvath et al., 1999; Brown et al., 2001), providing a mechanism by which the neuropeptide could influence REM sleep initiation. The diurnal variation in orexin levels, closely coupled to ongoing motor activity (Kiyashchenko et al., 2002), thus provides a basis for the variation in the expression of REM sleep throughout the nychthemeron. In this regard, it is interesting to note that McCarley and Massaquoi (1992), during development of a model of the REM sleep oscillator, postulated the existence of a circadian control factor that excited monoaminergic REM-off cells to prevent the occurrence of REM sleep and so influence the diurnal distribution of the state. Our data indicate that orexin could be a factor that plays such a role in REM sleep expression.

We conclude that this model of narcolepsy in the rat is likely to prove useful for research into the pathophysiology of the disorder and for the discovery and validation of pharmacological treatments based on orexin neurotransmission. With its ease of breeding propagation, in which one allele of the *orexin/ataxin-3* transgene is sufficient to yield the narcoleptic phenotype combined with extensive knowledge of neurophysiology and neuropharmacology in the rat, this model will be valuable in continuing investigations into narcolepsy.

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APPENDIX C

Cell Metabolism
Article

Enhanced Orexin Receptor-2 Signaling Prevents Diet-Induced Obesity and Improves Leptin Sensitivity

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SUMMARY

The hypothalamic orexin neuropeptide acutely promotes appetite, yet orexin deficiency in humans and mice is associated with obesity. Prolonged effects of increased orexin signaling upon energy homeostasis have not been fully characterized. Here, we examine the metabolic effects of orexin gain of function utilizing genetic and pharmacologic techniques in mice. Transgenic orexin overexpression confers resistance to high-fat diet-induced obesity and insulin insensitivity by promoting energy expenditure and reducing consumption. Genetic studies indicate that orexin receptor-2 (OX2R), rather than OX1R signaling, predominantly mediates this phenotype. Likewise, prolonged central administration of an OX2R-selective peptide agonist inhibits diet-induced obesity. While orexin overexpression enhances the anorectic-catabolic effects of central leptin administration, obese leptin-deficient mice are completely resistant to the metabolic effects of orexin overexpression or OX2R agonist infusion. We conclude that enhanced orexin-OX2R signaling confers resistance to diet-induced features of the metabolic syndrome through negative energy homeostasis and improved leptin sensitivity.

INTRODUCTION

Animals employ genetically and physiologically determined homeostatic mechanisms to prevent excess weight gain; however, this regulation may be circumvented by environmental and hedonic factors. The ready availability of palatable, calorically dense food and the reduced need for physical activity in modern life have resulted in a pandemic of obesity. Rodent models have elucidated the mechanisms and modulators of body weight homeostasis such as the fat tissue-derived satiety hormone leptin (Enriori et al., 2007; Myers et al., 2008). Leptin centrally regulates body weight by suppressing food intake and permitting energy expenditure. While absence of leptin in rodents causes hyperphagia, obesity, and diabetes, most human obesity is associated with hyperleptinemic leptin resistance.

Orexins (also known as hypocretins) are lateral hypothalamic neuropeptides that are upregulated with fasting and can acutely promote appetite when administered into the central nervous system (Sakurai et al., 1998). The two receptors for orexin, type 1 (OX1R) and type 2 (OX2R), show differential affinity for the products of the prepro-orexin gene, orexin-A and orexin-B (Sakurai et al., 1998). OX1R and OX2R exhibit distinct expression patterns, indicating distinct roles in behavior and metabolism. The arcuate nucleus of hypothalamus (ARH) is a point of convergence for both orexin and leptin signaling, which modulate the activities of neuropeptide regulators of food intake and metabolism such as neuropeptide Y (NPY), agouti-related peptide (AGRP), and pro-opiomelanocortin (POMC). Pathologic leptin resistance may be mediated by changes in second messengers, including the long form of leptin receptor (LEPR), downstream signal transducer and activator of transcription-3 (STAT3), or the feedback suppressor of cytokine signal-3 (SOCS3) (Horvath, 2005; Myers et al., 2008).

Central administration of orexin neuropeptides to rodents acutely promotes appetite, and prepro-orexin deficiency or postgestational ablation of orexin neurons in mice causes modest reductions in food intake. However, orexin-deficient mice also exhibit narcolepsy, inactivity, and obesity, indicating that orexin may exert an overall catabolic influence upon energy balance (Hara et al., 2001, 2005; Willie et al., 2001). Narcoleptic human individuals (the majority of which are orexin deficient) have also been reported to have greater body mass index and higher incidence of metabolic syndrome (Nishino, 2007). This effect of orexin upon energy balance may be primary, since orexin-deficient narcoleptic patients showed higher body mass index than otherwise clinically indistinguishable narcoleptics with normal orexin levels (Nishino et al., 2001).

As the conclusion that orexin promotes negative energy balance derives indirectly from loss-of-function studies, we utilized genetic and pharmacological methods to directly examine

whether increased orexin signaling promotes negative energy balance. First, using *CAG/orexin* transgenic mice (Mieda et al., 2004) that overproduce orexin neuropeptides from an ectopically expressed transgene, we examined the effects of constitutively increased orexin signaling upon diet-induced obesity by measuring adiposity, locomotor activity, and other metabolic parameters. To differentiate the role of each receptor pathway, we also examined the effects of the *CAG/orexin* transgene upon *OX1R* knockout mice ($OX1R^{-/-}$, Kisanuki et al., 2001) and *OX2R* knockout mice ($OX2R^{-/-}$, Willie et al., 2003). Results of genetic studies were then verified and extended pharmacologically using a selective agonist for OX2R. Finally, we sought to determine the effect of the *CAG/orexin* transgene upon leptin-deficient *ob/ob* mice, examined the sensitivity of *CAG/orexin* mice to central leptin administration, and investigated the effects of selective OX2R agonism upon *ob/ob* mice.

RESULTS

Expression of Orexin Peptide in *CAG/Orexin* Mouse

Previous results with *CAG/orexin* transgenic mice revealed multifold increases in both orexin-A and orexin-B peptides in whole brain extracts (Mieda et al., 2004). Immunohistochemical localization of orexin-A in the brain of *CAG/orexin* mice demonstrates ectopic peptide production in medial, basal, lateral, and suprachiasmatic hypothalamic nuclei, nucleus accumbens, globus pallidus, hippocampal formation, ventral tegmental area, and locus coerulus (Figures S1 and S2, Table S1). All of these locations have previously been implicated as participants in networks controlling various homeostatic, circadian, learned, and/or hedonistic aspects of food intake, taste preference, or energy homeostasis (Saper et al., 2002). Previous results demonstrated that *CAG/orexin* transgene insertion was sufficient to rescue the narcolepsy/cataplexy phenotype of mice lacking endogenous orexinergic neurons (Mieda et al., 2004). Thus, the *CAG/orexin* transgene produces functional peptides that can activate orexin receptors.

The physiological relevance of peripheral actions of orexins, if any, remains controversial (Heinonen et al., 2008). In spite of the use of a general promoter for orexin overexpression, we found that *CAG/orexin* mice exhibited ectopic orexin-A immunoreactivity in a limited set of peripheral tissues, including thyroid gland, adrenal cortex, and some pancreatic islets. No evidence of ectopic expression was encountered in other metabolic tissues such as brown and white adipose, liver, or skeletal muscle (Figure S3, Tables S2 and S3).

CAG/Orexin Mice are Resistant to Diet-Induced Obesity

To examine the effect of increased orexin on body weight, *CAG/orexin* transgenic mice and wild-type littermate mice were fed either a low- or a high-fat diet. In both male and female mice, the body weights of wild-type mice were significantly higher when fed a high-fat diet compared to a low-fat diet. However, mice overexpressing orexin did not show a significant difference in body weight growth between a low-fat diet and a high-fat diet (Figures 1A and 1B). Thus, wild-type mice are susceptible to diet-induced obesity, whereas *CAG/orexin* mice are quite resistant.

To determine which receptor pathway mediates the antiobesity effect of orexin overexpression, we crossed *CAG/orexin* transgenic mice to $OX1R^{-/-}$ and $OX2R^{-/-}$ lines. We compared the effects of isolated orexin-OX2R signaling (in $OX1R^{-/-}$ mice and $OX1R^{-/-}$; *CAG/orexin* mice) versus isolated orexin-OX1R signaling (in $OX2R^{-/-}$ mice and $OX2R^{-/-}$; *CAG/orexin* mice) upon growth curves. Figures 1C and 1D show that increased OX2R activation is sufficient to mediate the preponderance of resistance to diet-induced obesity. On the other hand, in both sexes, increased OX1R activation alone does not significantly protect from development of obesity (Figures 1E and 1F). Unlike differences in body weight, there were no significant differences in linear growth among the various genotypic groups (data not shown). Thus, OX2R signaling selectively mediates the antiobesity effect of orexin overexpression in mice challenged with a high-fat diet.

CAG/orexin-transgenic male mice were also resistant to aging-related adiposity, while wild-type male mice fed a low-fat diet showed continuous weight gain during aging (Figure 1A). In spite of similar growth curves before 18 weeks of age, the growth curve between 19 and 30 weeks of age of wild-type mice fed a low-fat diet was significantly larger than that of *CAG/orexin*-transgenic mice ($p = 0.0016$). Likewise, $OX1R^{-/-}$ male mice fed a low-fat diet showed larger body weight growth between 17 and 30 weeks of age than $OX1R^{-/-}$; *CAG/orexin* mice despite no significant difference in the growth curves before 16 weeks of age ($p = 0.036$). $OX2R^{-/-}$ male mice fed a low-fat diet showed larger body weight than $OX2R^{-/-}$; *CAG/orexin* mice through the whole observation period ($p = 0.005$); however, the fat mass and serum leptin of $OX2R^{-/-}$; *CAG/orexin* male mice were similar to those of $OX2R^{-/-}$ male mice (Figure 2).

CAG/Orexin Transgene Reduces Fat Mass and Leptin

Consistent with body weight data, at 28 weeks of age, *CAG/orexin* male mice showed a significant reduction of fat mass on a low-fat diet as compared with wild-type male mice (Figure 2A). The fat mass of *CAG/orexin* male mice and $OX1R^{-/-}$; *CAG/orexin* mice fed a high-fat diet was significantly less than those of wild-type mice and $OX1R^{-/-}$ mice, respectively, for both sexes (Figures 2A and 2B). There was no significant difference in fat mass between $OX2R^{-/-}$ mice and $OX2R^{-/-}$; *CAG/orexin* mice on both a low-fat and a high-fat diet for either sex. $OX2R^{-/-}$ male mice exhibited a significant tendency toward increased fat mass under high-fat conditions, and $OX2R^{-/-}$ female mice exhibited a mild but significant tendency toward increased fat mass under even low-fat conditions compared to wild-type mice, which is consistent with previously described adiposity of narcoleptic mice (Hara et al., 2001) and a physiological role of OX2R signaling in suppressing adiposity.

Next, we measured serum leptin, which typically correlates with fat mass. Concordance between fat mass and leptin levels was confirmed in each genotype. Specifically, the leptin levels of *CAG/orexin* mice and of $OX1R^{-/-}$; *CAG/orexin* mice were significantly lower than those of wild-type mice and of $OX1R^{-/-}$ mice fed a high-fat diet, respectively, whereas there was no significant difference in serum leptin levels between $OX2R^{-/-}$; *CAG/orexin* mice and $OX2R^{-/-}$ mice on both low-fat and high-fat diets for both sexes (Figures 2C and 2D). Compared to differences observed in fat mass, the *CAG/orexin* transgene was associated

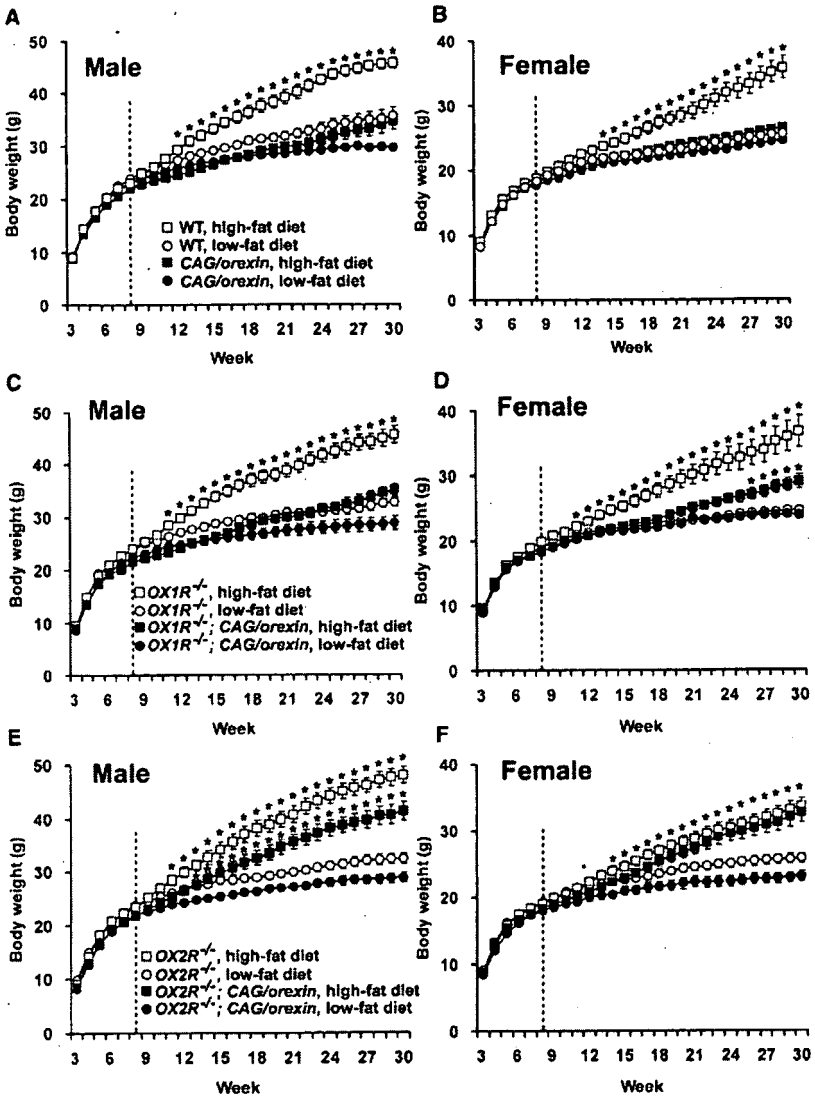


Figure 1. Growth Curves of Genetically Modified Mice Fed a Low- or High-Fat Diet

(A–F) Body weights of mice measured weekly from the age of 3 weeks to 30 weeks. A high-fat diet started at the age of 8 weeks (dotted line). The numbers of mice are 10–14 mice per group. * indicates significant difference between different diet condition for each genotypic group according to post hoc analysis at each time point. Significant differences in (F) for $OX2R^{-/-}$ mice and $OX2R^{-/-}$; *CAG/orexin* are indicated by one asterisk. Data are expressed as means ± SEM. Body weight curves of wild-type male (A) and female (B) mice on a high-fat diet were significantly higher than those of wild-type mice on a low-fat diet ($p < 0.0005$, both sexes). *CAG/orexin* mice did not differ significantly under low-fat and high-fat dietary conditions ($p = 0.51$ male; $p = 0.13$ female). Body weight curves of $OX1R^{-/-}$ male (C) and female (D) mice on a high-fat diet were significantly higher than those of $OX1R^{-/-}$ mice on a low-fat diet ($p < 0.0001$ male; $p < 0.01$ female). $OX1R^{-/-}$; *CAG/orexin* male mice did not differ significantly between low-fat and high-fat dietary conditions ($p = 0.21$). The body weights of $OX1R^{-/-}$; *CAG/orexin* female mice on a high-fat diet were significantly less than those of $OX1R^{-/-}$ mice on a high-fat diet ($p < 0.01$), in spite of no body weight difference between $OX1R^{-/-}$; *CAG/orexin* female mice and $OX1R^{-/-}$ female mice on a low-fat diet ($p = 0.50$). The body weight growths of $OX2R^{-/-}$ male (E) and female (F) mice on a high-fat diet were significantly higher than those of $OX2R^{-/-}$ mice on a low-fat diet ($p < 0.0001$ male; $p < 0.005$ female). Likewise, $OX2R^{-/-}$; *CAG/orexin* male (E) and female (F) mice showed significant weight gain on a high-fat diet compared to those on a low-fat diet ($p < 0.0005$ male; $p < 0.001$ female).

with small but significant reductions in lean mass of male mice having functional orexin receptors and those deficient in OX1R under high-fat conditions (Figure 2E). The *CAG/orexin* transgene was similarly associated with a significant mild reduction in lean mass of female mice having functional receptors under high-fat conditions, but a significant mild reduction of lean mass by the transgene under OX2R-deficient low-fat conditions was also observed (Figure 2F).

Increased Energy Expenditure of *CAG/Orexin* Mice

To explore the underlying cause of differential resistance to diet-induced obesity in mice overexpressing orexin, we housed mice from each genotypic group in metabolic cages in order to measure oxygen consumption, carbon dioxide production, and locomotor activity. The effective mass-corrected energy expenditures of *CAG/orexin* male mice and $OX1R^{-/-}$; *CAG/orexin* mice on a high-fat diet were consistently elevated over those of wild-type mice and $OX1R^{-/-}$ mice, respectively (Figures 3A, 3C, and 3G), while the energy expenditures of $OX2R^{-/-}$; *CAG/orexin* mice resembled those of $OX2R^{-/-}$ mice (Figures 3E and 3G). In contrast, we observed no consistent differences in respiratory quotient (RQ; an indirect indicator of lipid versus carbohydrate utilization) among different genotypic groups on a high-fat diet (Figures 3B, 3D, 3F, and 3H). The *CAG/orexin* transgene induced no differences in energy expenditure or RQ among any genotypic groups on a low-fat diet, regardless of the presence or absence of orexin receptors (Figure S4). Low-fat-fed $OX1R^{-/-}$ mice showed reduced energy expenditure compared to wild-type controls (Figure S4G). Importantly, *CAG/orexin* transgenic mice did not exhibit hyperactivity, regardless of diet or receptor status (Figure S5), although $OX2R^{-/-}$ mice fed a low-fat diet showed some reduced locomotion compared to wild-type mice (Figure S5G), which is consistent with previous data from narcoleptic mice (Hara et al., 2001, 2005). Basal core body temperature in *CAG/orexin* mice on a high-fat diet tended to be higher than in wild-type controls, but this difference did not reach significance (wild-type low-fat diet: 36.6°C ± 0.1°C; *CAG/orexin* low-fat

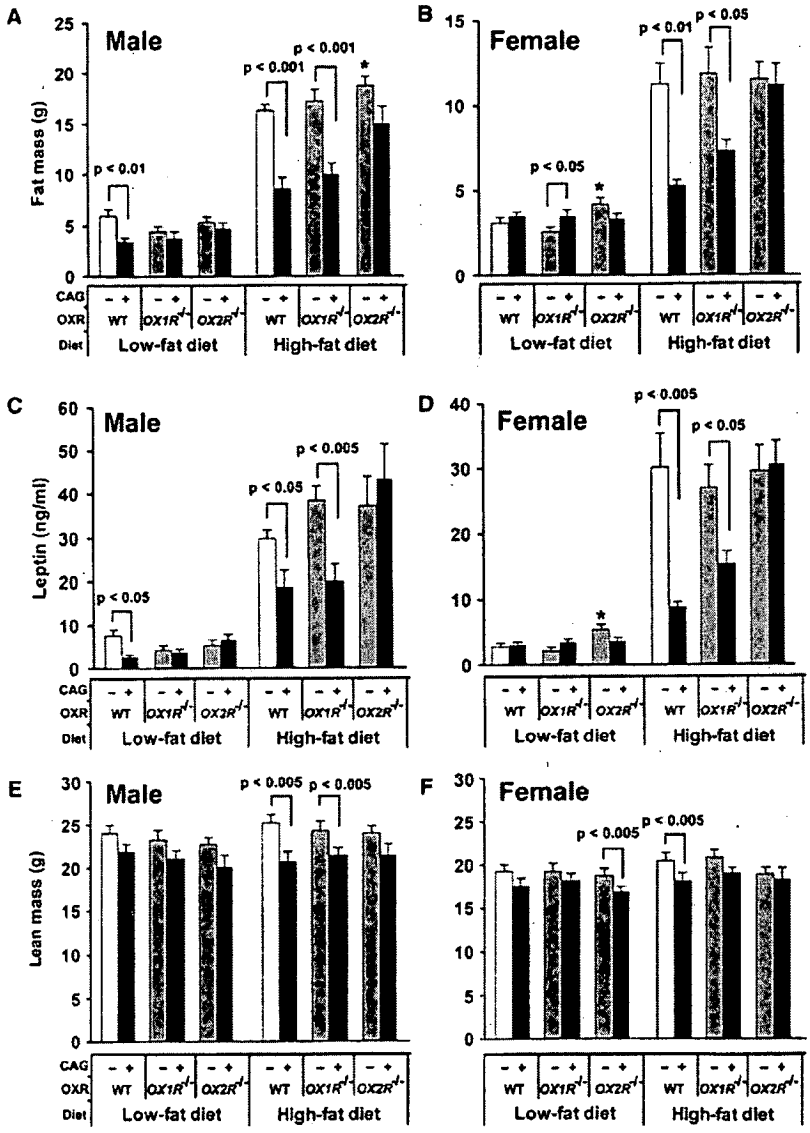


Figure 2. Fat Mass, Serum Leptin Levels, and Lean Mass of Orexin Signaling-Modified Mice

(A and B) The fat masses of male (A) and female (B) mice at 28 weeks of age on different diets.

(C and D) The serum leptin of male (C) and female (D) mice at 30 weeks of age on different diets.

(E and F) The lean masses of male (E) and female (F) mice at 28 weeks of age on different diets. * indicates significant ($p < 0.05$) increase compared to wild-type mice under the same food condition. The numbers of mice are 8–14 mice per group. Data are expressed as means ± SEM.

diet: 36.7°C ± 0.1°C; wild-type high-fat diet: 36.8°C ± 0.1°C; *CAG/orexin* high-fat diet: 37.0°C ± 0.1°C; n = 5–6).

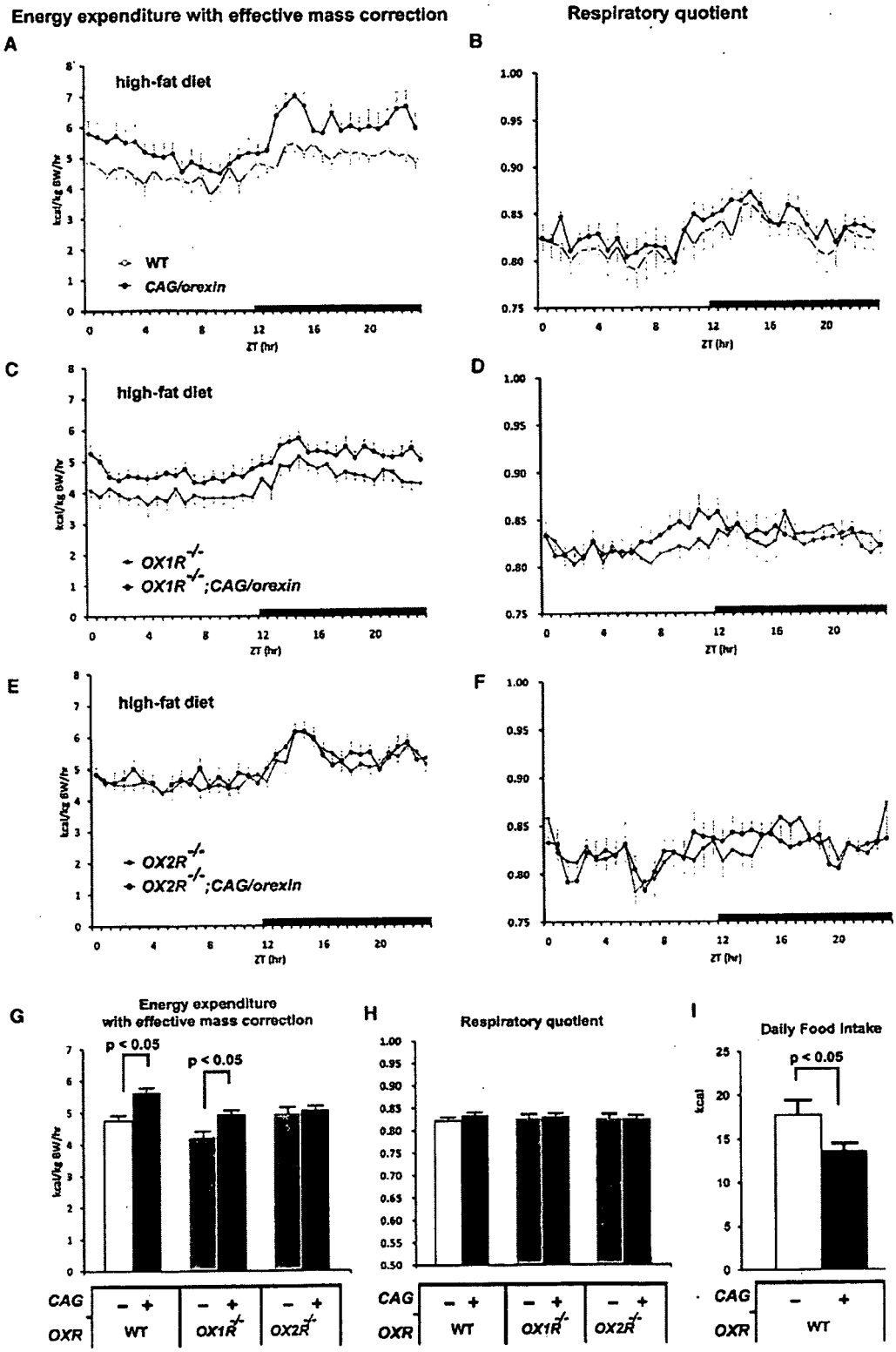
Both total high-fat diet intake for 14 days (Figure 3I) and body weight-adjusted daily food intake (data not shown) were significantly reduced in *CAG/orexin* mice compared to wild-type controls. Critically, this did not result from abnormal taste preferences: compared to wild-type mice, *CAG/orexin* and wild-type mice similarly exhibited greater preferences for high-fat over low-fat chow and for 10% sucrose over 1% sucrose solutions (Figure S6).

Glucose Metabolism of *CAG/Orexin* Mice

To examine the effect of orexin overexpression on glucose metabolism, we first measured blood glucose and serum insulin of fed mice at 30 weeks of age. When maintained on a low-fat diet, we observed no significant difference in fed glucose level among genotypes (Figure 4A). On a high-fat diet, however, wild-type mice exhibited hyperglycemia that is attenuated in *CAG/orexin*, *OX1R*$^{-/-}$, and *OX1R*$^{-/-}$; *CAG/orexin* mice, but not *OX2R*$^{-/-}$ or *OX2R*$^{-/-}$; *CAG/orexin* mice. Thus, the protective effect depends upon functional OX2R, but can be mediated by endogenous orexin levels even without orexin overexpression. Notably, these data also show that OX1R deficiency alone can prevent high-fat diet-induced hyperglycemia (see below).

Increased serum insulin levels with obesity or aging indicate mounting insulin resistance and sensitively predict deteriorating glucose control in human metabolic syndrome. When compared to wild-type mice, the *CAG/orexin* transgene reduced serum insulin levels on a low-fat diet and conferred protection from hyperinsulinemia on high-fat diet (Figure 4B). Notably, a similar protective effect occurred in *OX1R*$^{-/-}$ mice (Figure 4B), despite relative obesity under these conditions (Figures 1C, 1D, 2C, and 2D), suggesting that endogenous orexin-OX1R signaling can play a specific permissive role in development of hyperinsulinemia. However, the *CAG/orexin* transgene conferred protection from hyperinsulinemia upon all three genetic backgrounds on a high-fat diet, suggesting that both OX1R and OX2R mediate protective effects of orexin overexpression on insulin sensitivity.

We next examined the effects of orexin overexpression upon fasting glucose and glucose tolerance after glucose administration in mice. On a low-fat diet, orexin overexpression did not significantly affect glucose homeostasis ($p = 0.47$, Figure S7). On a high-fat diet, however, *CAG/orexin* mice exhibited significantly reduced basal fasting glucose levels as well as improved glucose tolerance at all time points tested, relative to wild-type controls (Figure 4C). Despite absence of basal differences in fasting serum glucose between *OX1R*$^{-/-}$ and *OX1R*$^{-/-}$; *CAG/orexin* mice, the *CAG/orexin* transgene conferred mild but significant improvements in glucose tolerance onto the *OX1R*$^{-/-}$


Energy expenditure with effective mass correction
Respiratory quotient
A
B
high-fat diet
WT
CAG/orexin
kcal/kg BW/hr
ZT (hr)
C
D
high-fat diet
OX1R-/-
OX1R-/-;CAG/orexin
E
F
high-fat diet
OX2R-/-
OX2R-/-;CAG/orexin
G
Energy expenditure with effective mass correction
p < 0.05
p < 0.05
H
Respiratory quotient
I
Daily Food Intake
p < 0.05
kcal
CAG
OXR
WT
OX1R-/-
OX2R-/-

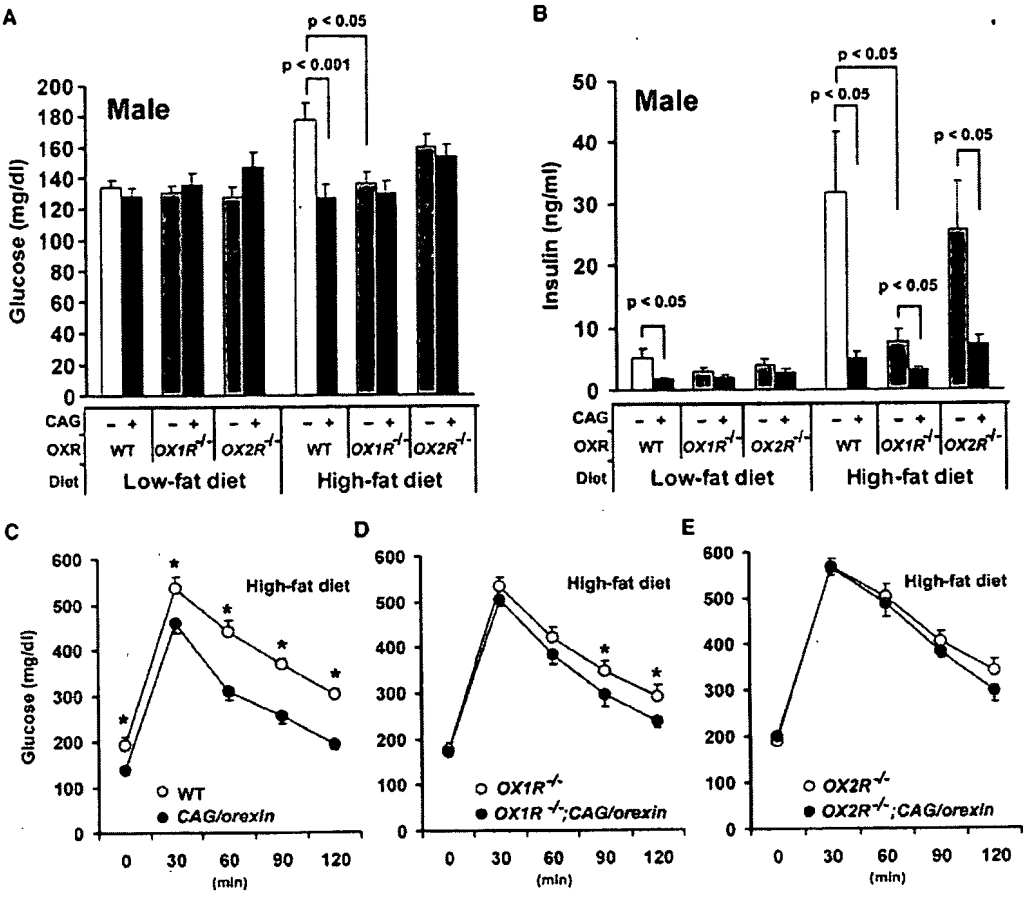


Figure 4. Glucose Metabolism of Orexin Signaling-Modified Mice on Different Fat Diets

(A) Blood glucose levels of orexin-related gene mutant mice on different fat diets.

(B) Serum insulin levels of orexin-related gene mutant mice on different fat diets.

(C) Glucose tolerance test showed that the blood glucose levels of *CAG/orexin* mice were significantly lower than those of wild-type littermate mice on a high-fat diet after the administration of glucose.

(D) Glucose tolerance test showed that the blood glucose levels of *OX1R*$^{-/-}$; *CAG/orexin* mice were significantly lower than those of *OX1R*$^{-/-}$ mice on a high-fat diet after the administration of glucose. Data are expressed as means ± SEM.

(E) Glucose tolerance test showed that there is no significant difference in the blood glucose levels between *OX2R*$^{-/-}$; *CAG/orexin* mice and *OX2R*$^{-/-}$ mice on a high-fat diet after the administration of glucose ($p = 0.33$). The numbers of mice are 8–14 mice (A and B) and 6–10 mice (C–E) per group. Data are expressed as means ± SEM (*$p < 0.05$).

background at later time points (Figure 4D). Improved glucose tolerance in the setting of reduced insulin levels indicates that the transgene confers improved insulin sensitivity. By contrast, we observed no significant differences in fasting glucose or glucose tolerance between *OX2R*$^{-/-}$ mice and *OX2R*$^{-/-}$; *CAG/orexin* mice (Figure 4E). Thus, while OX1R may also influence circulating insulin levels, orexin overexpression improves insulin sensitivity by a predominantly OX2R-dependent mechanism.

Effects of *CAG/Orexin* Transgene on Peripheral Tissues

Ectopic orexin production in thyroid tissue raises the possibility that abnormal activity of the thyroid axis contributes to leanness

Figure 3. The Metabolic Parameters of Orexin Signaling-Modified Mice on a High-Fat Diet

(A and B) The energy expenditure with effective mass correction (A) and respiratory quotient (B) sampled every 40 min over 24 hr of *CAG/orexin* mice and wild-type mice at 16–20 weeks of age.

(C and D) The energy expenditure with effective mass correction (C) and respiratory quotient (D) over 24 hr of *OX1R*$^{-/-}$; *CAG/orexin* mice and *OX1R*$^{-/-}$ mice.

(E and F) The energy expenditure with effective mass correction (E) and respiratory quotient (F) over 24 hr of *OX2R*$^{-/-}$; *CAG/orexin* mice and *OX2R*$^{-/-}$ mice.

(G and H) The averaged energy expenditure with effective mass correction (G) and respiratory quotient (H).

(I) Averaged daily high-fat diet intake of *CAG/orexin* mice and wild-type mice for 14 days. The numbers of mice are 6–9 mice per group. Data are expressed as means ± SEM.

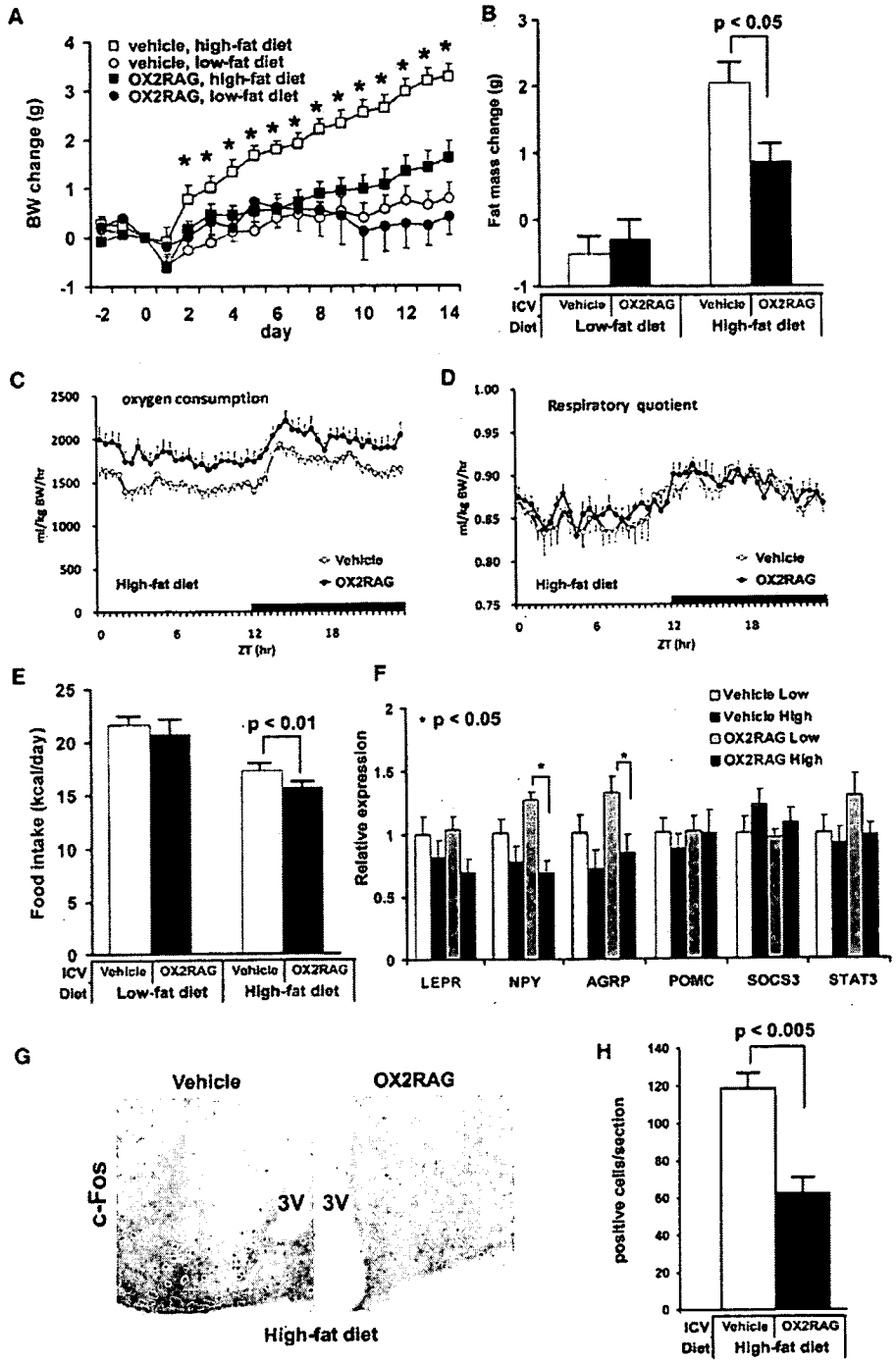


Figure 5. Effect of OX2R Selective Agonist on Diet-Induced Obesity

(A) The daily body weight changes of chronically ICV-injected mice. ICV and high-fat diet begin at day 0. The body weight growths of the OX2R selective agonist-injected mice (0.5 nmol/day) are significantly lower than those of vehicle-injected mice on a high-fat diet ($p < 0.0005$), whereas there is no significant difference in the body weight growth between them on a low-fat diet ($p = 0.45$).

(B) The fat mass change after the infusion of OX2R-selective agonist or vehicle for 14 days under a low- or high-fat diet.

(C) The oxygen consumption with effective mass correction of OX2R agonist-infused mice on a high-fat diet was higher than vehicle-infused mice ($p < 0.0005$, repeated ANOVA). Data were sampled every 30 min.

in *CAG/orexin* mice. We measured serum thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) on low- and high-fat diets. High-fat diet increased serum T3 and T4 levels of *CAG/orexin* mice to an extent similar to wild-type mice despite significant differences in adiposity between the groups (Figure S8). Serum TSH levels of *CAG/orexin* mice on a high-fat diet were significantly elevated over those on a low-fat diet, while a high-fat diet did not significantly affect serum TSH levels of wild-type mice. Importantly, the levels of serum TSH, T3, and T4 of *CAG/orexin* mice were similar to those of wild-type mice when maintained on a low-fat diet.

To determine whether increased energy expenditure of *CAG/orexin* mice was associated with increased mitochondrial uncoupling proteins, we examined mRNA levels of major uncoupling proteins in brown fat and skeletal muscle (Figure S9). High-fat diet resulted in comparable increases in *UCP1* mRNA in brown fat, but not skeletal muscle, in both genotypes. In contrast, *UCP2* and *UCP3* mRNA levels did not differ significantly by genotype or dietary condition, consistent with previous reports (Surwit et al., 1998).

Despite detection of ectopic orexin peptide in adrenal gland, *CAG/orexin* mice and wild-type mice had similar total daily urinary levels of epinephrine and norepinephrine and similar serum corticosterone levels (Figure S10). In addition, *CAG/orexin* transgene did not affect systolic blood pressure on either a low-fat or high-fat diet (Figure S10).

OX2R Agonist Prevents Diet-Induced Obesity

Our genetic studies implicate the OX2R pathway as mediator of the effects of orexin overexpression upon energy homeostasis. To further test the hypothesis that central enhancement of orexin-OX2R signaling confers resistance to diet-induced obesity, an OX2R selective agonist [Ala11, D-Leu15] Orexin-B (Asahi et al., 2003) was continuously infused in the lateral ventricles of wild-type mice for 14 days. The administration of the OX2R selective agonist suppressed weight gain on a high-fat diet without altering weight homeostasis on a low-fat diet (Figure 5A). Importantly, the OX2R selective agonist had no obvious effect upon OX2R-deficient mice on a high-fat diet ($n = 4$, weight gain 3.33 ± 0.61 g, $p = 0.67$), verifying the specificity of the agonist in vivo. Following 14 days, the agonist-infused wild-type mice gained significantly less fat mass than did the vehicle-injected mice on a high-fat diet, and no effect was observed on a low-fat diet (Figure 5B). When centrally infused mice fed a high-fat diet were monitored in metabolic chambers, OX2R agonist infusions resulted in consistently greater energy expenditures (Figure 5C), but not RQs (Figure 5D) or locomotor activity (data not shown), over vehicle-infused controls.

As sleep/wake disturbances could affect food intake and energy expenditure, we recorded EEG/EMG signals during central OX2R agonist or vehicle infusions. Mice receiving OX2R agonist exhibited total wake or sleep times during both light and dark phases that closely resembled vehicle controls, irrespective of dietary condition (Figure S11). As predicted from previous studies (Willie et al., 2003), OX2R agonism continued to promote consolidation of behavioral states, as demonstrated by increased wake and NREM episode durations in mice maintained on a low-fat diet. As this consolidation was not evident under high-fat-fed conditions, sleep/wake change cannot be the primary cause in metabolic effects of enhanced orexin signaling observed predominantly under high-fat conditions.

We observed an expected homeostatic reduction of food intake in mice maintained on a high-fat diet compared to a low-fat diet (West et al., 1992), and administration of the agonist significantly enhanced this effect by further suppressing food intake selectively in mice fed a high-fat diet (Figure 5E). After 14 days of OX2R agonist administration, we observed reduced hypothalamic mRNA expression of orexigenic factors *NPY* and *AGRP* on a high-fat diet compared to those on a low-fat diet (Figure 5F). Indeed, the number of c-Fos-positive cells in ARH region was significantly reduced in OX2R agonist-administered mice on a high-fat diet (Figures 5G and 5H). The reduction of c-Fos-positive cell number was particularly notable in the ventromedial aspect of ARH (Figure 5G), in which orexigenic NPY/AGRP neurons are located (Horvath, 2005), consistent with the observed reduction in food intake and in *NPY/AGRP* mRNAs we observed under this condition.

Leptin Mediates Antiobesity Effects of Orexin

Leptin negatively regulates body weight, suppresses food intake, and increases energy expenditure by inhibiting NPY/AGRP neurons and activating POMC neurons of ARH. Diet-induced obesity is associated with leptin resistance resulting from signal transduction abnormalities in ARH (Myers et al., 2008). OX2R is highly expressed in ARH, and the effects of circulating leptin upon ARH resemble some effects of increased orexin-OX2R signaling that we observed. We hypothesized, therefore, that leptin signaling mediates some of the metabolic effects of orexin. To examine the consequences of orexin signaling enhancement on mice in the absence of leptin activity, we crossed *CAG/orexin*-transgenic and leptin-deficient *ob/ob* lines. Remarkably, the *CAG/orexin* transgene had no impact upon weight gain or fat mass of leptin-deficient *ob/ob* mice (Figures 6A and 6B), suggesting that, indeed, the antiobesity effect of *CAG/orexin* depends upon leptin activity. We then centrally administered OX2R agonist to *ob/ob* mice and similarly found no significant effect upon weight gain under low- or high-fat dietary conditions (Figure 6C). We also observed no effect of OX2R agonist compared to vehicle administration upon core body temperature of *ob/ob* mice (data not shown).

We directly examined whether orexin overexpression alters sensitivity to leptin. Leptin was continuously administered in

(D) The respiratory quotient of OX2R agonist-infused mice on a high-fat diet was similar to that of vehicle-infused mice.
(E) The average daily food intake of mice injected with the OX2R selective agonist or vehicle on different fat diets during 14 days.
(F) Hypothalamic gene expressions at the end of OX2R selective agonist administration are determined using q-PCR. Gene expressions are normalized by GAPDH.
(G) Immunostaining for c-Fos in ARH region of mouse on a high-fat diet during central administration of OX2R agonist or vehicle; 3V, third ventricle.
(H) The number of c-Fos-positive cells in ARH region. The numbers of mice per group are 7–14 mice for (A), (B), and (C); 6–7 mice for (D), (G), and (H); and 5–6 mice for (E) and (F). Data are expressed as means ± SEM.

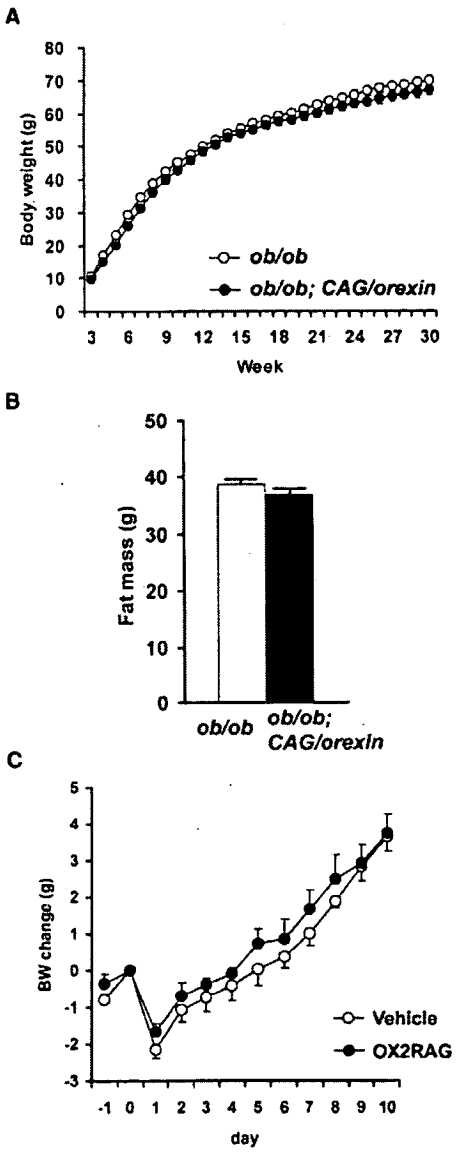


Figure 6. No Effect of Orexin Overexpression on the Weight Gain of *ob/ob* Mouse

(A) The body weight growth of *ob/ob*; *CAG/orexin* male mice was similar to that of *ob/ob* male mice ($p = 0.76$).

(B) Fat mass of 28-week-old *ob/ob* male mice with or without *CAG/orexin* transgene. There is no significant difference in fat mass for both male and female. There were 13–20 mice per group.

(C) The body weight growth of OX2R agonist-infused *ob/ob* mice was similar to that of vehicle-injected *ob/ob* mice maintained on a low-fat and a high-fat diet. Data are expressed as means ± SEM.

the lateral ventricles of *CAG/orexin* and wild-type littermate pairs. Mice (3–4 months old) were maintained on a low-fat diet in order to initially match as to body weight (WT 29.7 ± 3.6 ng/μl and *CAG/orexin* 27.0 ± 3.1 ng/μl). Both wild-type mice and *CAG/orexin* mice lost weight during the administration of leptin, but *CAG/orexin* mice showed significantly enhanced weight loss and anorexia compared to wild-type mice on a low-fat diet (Figures 7A and 7B), indicating that increased orexin signaling is associated with a more leptin-sensitive state.

Compared to control wild-type mice, 14 days of central leptin administration resulted in basal hypothalamic expression levels of *NPY* and *AGRP* and an expected induction of *POMC* mRNA (Figure 7C). In contrast, under basal conditions, *CAG/orexin* transgenic mice showed increased expression of *NPY* and *AGRP*, but not *POMC* mRNA. While we detected no significant changes in the expression of LEPR, SOCS3, or STAT3 gene products, the overall profile of altered hypothalamic gene expression we detected is consistent with the physiological state of anorexia and weight loss observed in mice undergoing leptin administration.

DISCUSSION

When challenged with a high-fat diet, *CAG/orexin* mice maintain elevated energy expenditure, decreased food intake, and resistance to diet-induced obesity, hyperleptinemia, and hyperinsulinemia, although these mice show normal adiposity and energy homeostasis under a low-fat diet. Molecular genetic dissection of the metabolic phenotype utilizing *CAG/orexin*; $OX1R^{-/-}$ and *CAG/orexin*; $OX2R^{-/-}$ mice indicated that OX2R predominantly mediates these antiadipogenic effects and improves insulin sensitivity. Central infusion of an OX2R agonist confirms the role of central orexin-OX2R signaling in protection from high-fat diet-induced obesity. Furthermore, the antiadipogenic effects of genetic or pharmacologic enhancement of orexin signaling require leptin, and *CAG/orexin* mice exhibit increased sensitivity to exogenous leptin infusion.

Technical Considerations Regarding *CAG/Orexin* Transgene

The *CAG* promoter is a universal, constitutively active promoter, yet ectopic orexin production was restricted to a limited number of tissues, likely due to the necessity of lineage-specific enzymatic machinery required for neuropeptide production. Likewise, immunohistochemical localization of orexin-A peptide does not demonstrate homogenous presence of the antigen throughout brain parenchyma, but restricted presence at specific brain regions. In *CAG/orexin* mice, the appearance and number of strongly orexin-A-positive neurons in LHA (endogenous orexin cells) and the density of orexin-A-positive fibers in brain regions to which endogenous orexin neurons normally project resemble wild-type mice (Figure S2). Additionally, we observe a diffuse background ectopic orexin-A immunoreactivity, especially in the medial basal hypothalamus, including the ARH (Figures S1 and S2). Ectopically expressed orexin confers physiologic signaling, which is demonstrated by our previous result that the *CAG/orexin* transgene rescues the narcolepsy-cataplexy phenotype (Mieda et al., 2004). Moreover, dependency of the *CAG/orexin* phenotype upon the intact OX2R gene demonstrates that the metabolic phenotype of *CAG/orexin* mice is not an artifact, but the physiological effect of increased orexin-OX2R signaling.

Although we cannot rule out a contribution of ectopic orexin production in the thyroid, adrenal medulla, and pancreatic islets to the phenotype of *CAG/orexin* mice, we found no indication of

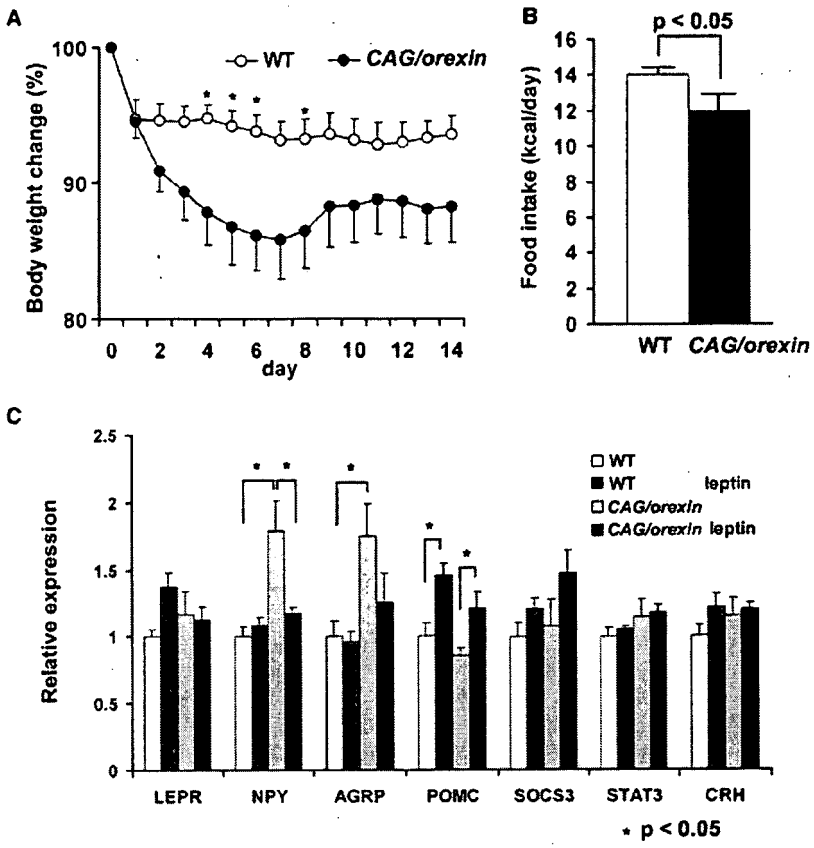


Figure 7. Increased Sensitivity of Orexin Overexpression Mouse to Leptin

(A) The body weight changes during chronic ICV injection of leptin (2 μg/day) for 14 days. *CAG/orexin* mice on a low-fat diet show larger weight loss compared with wild-type mice ($p < 0.05$).

(B) Daily food intake of *CAG/orexin* mice during chronic injection of leptin is smaller than that of wild-type mice.

(C) Hypothalamic gene expressions at the end of leptin administration. There were 6–8 mice per group. Data are expressed as means ± SEM (*$p < 0.05$).

primary peripheral endocrine disturbance in *CAG/orexin* mice. *CAG/orexin* mice have normal levels of serum TSH, T3, T4, corticosteroid, and urinary catecholamines, and we observed improved glucose metabolism consistent with increased insulin sensitivity and reduced leptin levels that correspond in the expected manner to reduced adiposity. Furthermore, replication of antiadipogenic effects with central OX2R agonist administration suggests that the metabolic effects of enhanced orexin signaling originate centrally.

Orexin Signaling Promotes Negative Energy Balance

Energy balance is a function of caloric intake and energy expenditure, and our data indicate that both orexin overexpression and OX2R agonist infusion increase energy expenditure and further suppress consumption of a high-fat diet, providing the mechanistic rationale for observed resistance to adiposity. While this result seems at odds with the well-documented acute pharmacologic orexigenic activity of orexin (Sakurai et al., 1998; Willie et al., 2001), chronic central administration of orexin-A does not support increased food consumption or anabolism in rats (Yamanaka et al., 1999). This suggests that the acute appetite-promoting effects of orexin peptides may be temporary, or progressively overwhelmed by counterregulatory mechanisms that oppose weight gain.

Low-fat-fed mice carrying the *CAG/orexin* transgene or treated with OX2R agonist for 2 weeks demonstrated elevated expression of the orexigenic genes *NPY* and *AGRP*. These changes could represent a direct effect, as orexin acutely stimulates neurons of the ARH when microinjected (van den Top et al., 2004; Yamanaka et al., 2000), and orexin-stimulated food consumption depends pharmacologically upon NPY signaling (Yamanaka et al., 2000). An alternative mechanism for upregulated *NPY* and *AGRP* transcription could be a compensatory response to relative negative energy balance, since we noted significant differences from controls only when mice were maintained on calorie-poor rather than calorie-dense chow.

Despite differences in orexigenic effects across different experimental paradigms, consistent and unifying results from pharmacologic and genetic studies indicate that orexin gain of function promotes energy expenditure while loss of function promotes energy conservation. Just as the orexin system is believed to orchestrate disparate circuits of the ascending arousal system to maintain a consolidated state of arousal, it may also normally serve to consolidate the activity in parallel reward and metabolic networks that control behavioral and homeostatic responses to support energy expenditure. The exact peripheral (downstream) mechanisms for the orexin-mediated increases of energy consumption remain unclear. Although we did not detect significant increase in urinary catecholamines or basal blood pressure, the data do not exclude the possibility of a subtly increased sympathetic tone in certain peripheral tissues. Indeed, we speculate that the sympathetic pathways are one of likely downstream mechanisms for the increased metabolic rate under enhanced orexin signaling.

Interactions of Orexin and Leptin Signals

Antiadipogenic effects of orexin-OX2R signaling require the presence of leptin, and orexin-overexpressing mice showed increased sensitivity to catabolic-anorectic effects of exogenous leptin. These findings suggest that leptin mediates the suppressive effect of enhanced OX2R signaling on diet-induced obesity. Leptin-responsive neurons are found in ARH, VMH, DMH, LHA, and tuberomamillary nucleus of the hypothalamus (Elmquist,

2000). These nuclei receive orexin innervations, express high levels of OX2R, and exhibit ectopic orexin immunostaining in *CAG/orexin*-transgenic mice. Among these, ARH is a particularly critical nexus for body weight regulation that monitors peripheral energy storages and enteral feeding status through integration of circulating leptin and insulin, metabolites, and vagal relays. Through outputs to other hypothalamic and brainstem sites, ARH modulates the thresholds, triggering drives to eat and expend energy, and it influences insulin secretion and sensitivity (Horvath, 2005; Coppari et al., 2005; Myers et al., 2008). Moreover, ARH harbors cellular abnormalities underlying acquired leptin resistance (Kievit et al., 2006), and reduced leptin sensitivity in ARH has been causally linked with diet-induced obesity (Enriori et al., 2007). While ARH neurons project to orexin neurons of the LHA, ARH receives dense reciprocal orexin fiber innervation and expresses mainly OX2R receptor (Cluderay et al., 2002; Peyron et al., 1998; Marcus et al., 2001). Acute microinjections of orexin-A into ARH increase oxygen consumption and body temperature under anesthesia (Wang et al., 2003).

The mechanism by which orexin and leptin signals interact remains unclear. Neurons expressing both LEPR and OX2R may have convergent intracellular second messenger signaling, including extracellular factor-regulated kinase (ERK) and the Janus kinase JAK2/STAT3 pathways (Myers et al., 2008; Zhu et al., 2003). In ARH, leptin-responsive neurons such as those expressing NPY/AGRP are directly excited by orexin while POMC neurons are directly inhibited by orexin (Muroya et al., 2004), but inhibitory GABAergic interneurons in ARH may also be activated via postsynaptic OX2R (Burdakov et al., 2003), predicting complexity in up- or downregulation of these circuits. Fos immunostaining reveals reduced neuronal activity in a population of ARH neurons following 2 weeks of OX2R agonist administration on a high-fat diet, but the true molecular identity of these cells and the direct versus indirect nature of this effect requires investigation.

Orexin neurons could directly sense lipids through kinetics of fatty acid metabolites to alter feeding behavior and energy homeostasis. However, we observed *CAG/orexin*-transgenic mice to be also resistant to aging-associated adiposity, even when maintained on a low-fat diet, and we detected no significant differences in circulating cholesterol or fatty acids among genotypes or dietary conditions (data not shown). Therefore, abnormal kinetics of hypothalamic fatty acid metabolism alone is unlikely to explain the obesity-resistant *CAG/orexin* phenotype. Furthermore, endogenous orexin neurons are themselves unlikely to play a crucial lipid-sensing role in energy homeostasis, as *CAG/orexin* mice in which endogenous orexin neurons have been selectively eliminated remain lean (J.T.W. and T.S., unpublished data).

Potential Role of OX1R on Glucose Metabolism

The unifying scheme in the present study is that OX2R, but not OX1R, is the primary receptor that mediates the beneficial effects of orexin gain of function under a high-fat diet. The observed improvements in glucose metabolism and insulin sensitivity could largely be explained by the OX2R-mediated reduction of body adiposity. However, there is a notable exception: we observed that OX1R deficiency alone, without orexin overexpression, can improve glycemia and insulin sensitivity on a high-fat diet (Figures 4A and 4B), despite the fact that $OX1R^{-/-}$ and wild-type mice are similarly obese under a high-fat diet (Figures 1C and 2C). This suggests that endogenous levels of orexin acting on OX1R may, in part, mediate the deleterious effects of high-fat diet on glucose metabolism. Indeed, OX1R is expressed in the solitary tract nucleus and dorsal motor nucleus of the vagus (Marcus et al., 2001), which participates in the regulation of hepatic glucose production (Pocai et al., 2005). Although OX1R is also detected in beta cells of pancreatic islets, a role for orexin, if any, in the pancreatic islet is controversial (Heinonen et al., 2008). At any rate, under orexin overexpression, the OX2R-mediated effects prevail, and the presence or absence of OX1R does not affect (the improvement of) glycemia or insulinemia. The specific role of OX1R on glucose regulation merits further investigation.

Therapeutic Implications

The robust innervation by the orexin system of the whole brain and the multiple phenotypic aspects of orexin-deficient animals such as cataplexy, attenuated morphine dependence, and diminished stress response has led to conceptualization of the orexin system as a hypothalamic output pathway controlling arousal, motivational behavior, and autonomic responses (Chemelli et al., 1999; Sakurai, 2007). Our results demonstrate that orexin signaling also has the capacity to primarily promote energy expenditure via leptin sensitization. Augmentation of OX2R signaling or its downstream targets beneficially alters hypothalamic setpoints controlling metabolic rate, food intake, and leptin and insulin sensitivity. Similar interventions in humans might prevent or reverse the effects of consumption of calorie-dense food that promote or maintain pathological adiposity and metabolic syndrome. From a therapeutic standpoint, it is important to note that orexin gain of function did not overtly alter the basal blood pressure, or the thyroid, glucocorticoid, and catecholamine statuses in our models.

While continuous orexin gain of function did not induce locomotor hyperactivity or perturb overall amounts of sleep and wakefulness in *CAG/orexin* mice or OX2R agonist-infused mice, further sleep/wake characterization of these models is warranted. The metabolic syndrome is a disorder not only of obesity and insulin insensitivity, but possibly also inactivity, sleep/wake disturbances, and comorbid depression (Fabricatore and Wadden, 2006). Daytime administration of an OX2R agonist to such individuals could have multiple beneficial effects by maintaining elevated metabolic rate while also promoting daytime wakefulness and consolidating sleep/wake states. The orexin system has emerged as a key target for therapeutic intervention in disorders associated with hypothalamic dysfunction, including not only narcolepsy and hypersomnia, but now also the metabolic syndrome.

EXPERIMENTAL PROCEDURES

Animals

All mice were backcrossed more than ten generations to the C57BL/6J strain. In *CAG/orexin*-transgenic mice, the expression of prepro-orexin is controlled by the chimeric *CAG* promoter constructed from the chicken β-actin promoter and the cytomegalovirus immediate early gene enhancer (Mieda et al., 2004). Each genotypic group was compared by pairing of littermates as follows: wild-type with *CAG/orexin*, $OX1R^{-/-}$ (Kisanuki et al., 2001) with $OX1R^{-/-}$; *CAG/orexin*,

OX2R$^{-/-}$ (Willie et al., 2003) with *OX2R*$^{-/-}$; *CAG/orexin*, *ob/ob* with *ob/ob*; *CAG/orexin*. *Ob/ob* (Zhang et al., 1994) mice were obtained from Jackson Laboratory. Mice were provided food and water ad libitum, maintained on a 12 hr light/dark cycle at all times, and housed at two or three mice per cage under controlled temperature and humidity, unless otherwise specified. All procedures were approved by the appropriate Institutional animal care and use committees and were carried out in strict accordance with NIH guidelines.

Body Weight Study

For diet-induced obesity, all mice were fed a low-fat diet (standard chow 8664 F6 Rodent Diet; Harlan Teklad) until 8 weeks of age. At 8 weeks of age, mice were assigned randomly to either a low-fat or a high-fat diet (D12451; Research Diets). A low-fat diet provided 4.1 kcal/g of energy (67% carbohydrate, 20% protein, and 13% fat). A high-fat diet provided 4.7 kcal/g of energy (35% carbohydrate, 20% protein, and 45% fat). Body weight was measured weekly until 30 weeks of age. At 28 weeks of age, mice were subjected to NMR (Minispec NMR Analyzer; Bruker) to measure fat and lean mass per the manufacturer's instructions. At 30 weeks of age, mice were euthanized to collect blood and measure blood glucose. Serum was collected from centrifuged blood and stored at −80°C until use. *Ob/ob* and *ob/ob; CAG/orexin* mice were fed a low-fat diet until 30 weeks of age.

Blood Analysis

For analysis of serum, we used Mouse Leptin and Ultra Sensitive Rat Insulin ELISA kits with Mouse Insulin Standard (Crystal Chem). Whole blood glucose levels were measured using a standard clinical glucometer (Elite; Bayer). For glucose tolerance tests, 21- to 25-week-old mice were fasted for 12 hr from ZT16 and then injected with glucose (1.5 g/kg of body weight, i.p.) at ZT4. Tail blood was collected at 0, 15, 30, 60, and 90 min after injection.

Metabolic Cage Study

Indirect calorimetry and locomotor data were simultaneously measured using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). For genetic studies, 16- to 20-week-old animals were individually housed in calorimeter chambers, and 3 days of data collection followed a 4 day acclimatization period. For pharmacologic studies, ICV cannulation surgery was performed at 10–12 weeks of age, and high-fat feeding initiated in an acclimatization chamber. After 4 days of acclimatization, each animal was housed in a metabolic chamber for 4.5 days. We calculated metabolic parameters based on the following equations:

$$RQ = CO_2 \text{ production/oxygen consumption.}$$

$$\text{Raw Energy Expenditure (REE)} = (3.815 + 1.232 \times RQ) \times \text{oxygen consumption.}$$

$$\text{Energy Expenditure with Effective Mass Correction} = \text{REE}/(\text{weight/mass unit})^{\text{effective mass factor}}. \text{ Effective mass factor} = 0.75.$$

Chronic ICV Injection

Three- to four-month-old male C57B/6J mice were single-housed 1 week before surgery and fed a low-fat diet. Mice were anesthetized with ketamine and xylazine (100 mg/Kg and 10 mg/Kg, respectively, i.p.). A cannula (Brain Infusion Kit III; Alzet) was implanted into the right lateral ventricle (0.3 mm posterior from the bregma, 0.9 mm lateral from the midline, and 2.4 mm from the surface of skull) using standard sterile stereotactic techniques. An osmotic minipump (model 2001; Alzet) was attached to the cannula and implanted in the subcutaneous space during the same surgical session. The OX2R selective agonist ([Ala11, D-Leu15] Orexin-B; American Peptide) (Asahi et al., 2003) or vehicle was continuously injected in the lateral ventricle for 14 days (0.5 nmol/day). The agonist was diluted with vehicle (Dulbecco's PBS; Sigma) immediately before use. At the day of surgery, the implanted mice were randomly assigned to a low-fat diet or a high-fat diet. Body weight and food intake were monitored daily for 14 days, and fat mass was detected by NMR immediately after surgery and again at day 14. Twelve-week-old *ob/ob* male mice were used for chronic ICV infusion of OX2R agonist for 10 days as described above. For leptin administration experiments, weight-matched 3- to 4-month-old *CAG/orexin* and wild-type littermates were continuously injected with leptin (2 μg/day; PreproTech), as described above, while maintained on a low-fat diet. Body weight and food intake were monitored daily for 14 days.

Quantitative PCR

Hypothalamus was dissected coronally under microscope from the optic chiasm to the mamillary bodies. The thick coronal section was further trimmed bilaterally at 1 mm from the midline and dorsally at 1.5 mm from the ventral surface. This dissected tissue included ARH, VMH, DMH, PVN, anterior hypothalamic area, and a part of LHA. Total RNA was isolated using RNeasy Mini Kit and used for cDNA synthesis by random hexamer and Omniscript Reverse Transcriptase (QIAGEN). Real-time quantitative PCR reactions were performed on cDNA with ABI Prism 7000 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's manual. *GAPDH* mRNA level was used for normalization.

Immunohistochemistry

After 2 weeks of continuous administration of OX2R agonist or PBS, mice on a high-fat diet were harvested during early dark phase under a red light. The immunohistochemistry for c-Fos was performed using free-floating method, as described previously (Chemelli et al., 1999), utilizing anti-c-Fos polyclonal antisera (Ab-5; Oncogene). Fos-positive cells in ARH region of two sections per animal were counted by an observed blinded-to-treatment group.

Data Analysis

Body weight growths and glucose tolerance tests were examined using repeated-measure analysis of variance (ANOVA) followed by Tukey's post hoc test, except where otherwise specified.

SUPPLEMENTAL DATA

Supplemental Data include 11 figures, two tables, and Supplementary References and can be found online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(08)00351-3.

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